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**ENDOTHELIAL CELL FUNCTION:
ROLE OF INTRACELLULAR SIGNALLING.**

A thesis submitted for the degree of

Doctor of Philosophy

in the University of Glasgow

by

Kevin William Buchan

**Department of Pharmacology,
University of Glasgow,
September, 1991.**

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SUMMARY

A. Regulation of calcium mobilisation in bovine aortic endothelial cells

1. In the presence of 1.8mM extracellular calcium, bradykinin (0.3nM-100nM), adenosine triphosphate (ATP; 0.3 μ M-100 μ M) and thrombin (0.03U ml⁻¹-3U ml⁻¹) each induced biphasic elevations of intracellular free calcium ([Ca²⁺]_i) in bovine aortic endothelial cells (BAEC), consisting of a large, initial transient component, which peaked within 30 seconds, followed by a lower, sustained plateau phase.

2. Platelet-activating factor (up to 120nM), histamine (up to 10 μ M) and lipopolysaccharide (up to 10 μ g ml⁻¹) each had no effect on [Ca²⁺]_i in BAEC.

3. In the presence of 1.8mM extracellular calcium, treatment of endothelial cells with the calcium influx blocker, nickel chloride (4mM), had no effect on basal [Ca²⁺]_i or on the magnitude of the bradykinin-induced transient elevation of [Ca²⁺]_i, but abolished the plateau phase.

4. When BAEC were bathed in nominally calcium-free solution, containing 0.5mM EGTA, bradykinin and ATP each induced only a transient elevation of [Ca²⁺]_i: the magnitude of this component was significantly smaller than that obtained in the presence of extracellular calcium, and the plateau phase was abolished. In the continued presence of bradykinin or ATP, re-addition of extracellular calcium, to achieve a level of around 1.8mM, resulted in the induction of a large, initial transient component, followed by a lower, sustained component. Procaine (1mM) had no effect on the large, transient component, suggesting that calcium-induced calcium release is not involved in the generation of this component.

5. In the presence of 1mM extracellular calcium, treatment with the calcium chelator, EGTA (2mM; 1min), slightly reduced basal $[Ca^{2+}]_i$ and significantly reduced the magnitude of the bradykinin-induced transient elevation of $[Ca^{2+}]_i$. Increasing the exposure time to 10min or the concentration of EGTA to 5mM resulted in no further reduction in the magnitude of the bradykinin-induced transient component.

6. An attempt was made to selectively inhibit release of calcium from intracellular stores. In the presence of 1.8mM extracellular calcium, treatment of BAEC with the putative inhibitor of intracellular calcium release, 3,4,5-trimethoxybenzoic acid-8-(diethylamino) octyl ester (TMB-8; 0.1mM) increased basal $[Ca^{2+}]_i$ slightly, but had no effect on either component of the bradykinin-induced biphasic elevation of $[Ca^{2+}]_i$. To determine the effects of TMB-8 on only the intracellular release component, BAEC were bathed in nominally calcium-free solution, in the presence of EGTA (0.5mM). TMB-8 (0.1mM) had no effect on basal $[Ca^{2+}]_i$ or the bradykinin-induced transient elevation of $[Ca^{2+}]_i$. These findings suggest that TMB-8 does not inhibit release of calcium from intracellular stores.

7. An attempt was made to determine whether removal of extracellular calcium results in the depletion of intracellular calcium stores. The elevation of $[Ca^{2+}]_i$ induced by caffeine (5mM) was almost completely abolished when BAEC were bathed in nominally calcium-free solution containing 0.5mM EGTA, suggesting that depletion of these stores does occur.

8. In the presence of 1.8mM extracellular calcium, addition of potassium chloride (KCl; 30mM and 60mM) had no effect on basal $[Ca^{2+}]_i$. However, 60mM KCl, but not 30mM KCl, reduced the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by bradykinin (10nM). Addition of KCl(30mM) during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by bradykinin (10nM),

thrombin (1U ml^{-1}) and ATP ($30\mu\text{M}$), resulted in a fall in $[\text{Ca}^{2+}]_i$ which was not well maintained. Hence, calcium entry in BAEC does not occur through voltage-operated channels, although the effects of membrane depolarisation on agonist-induced calcium mobilisation appear to be complex.

9. It has been proposed that activation of protein kinase C may inhibit the release of EDRF by inhibition of agonist-induced calcium mobilisation. Pre-treatment of BAEC with an activator of protein kinase C, 4β -phorbol 12-myristate 13-acetate (PMA; 100nM , 5min) reduced the magnitude of the initial transient elevation of $[\text{Ca}^{2+}]_i$ induced by thrombin (1U ml^{-1}), and by low concentrations of bradykinin (1nM) or ATP ($0.3\mu\text{M}$, $3\mu\text{M}$), but not by higher concentrations of the latter two agonists. Addition of PMA (100nM) during the plateau phase of the increase in $[\text{Ca}^{2+}]_i$ induced by thrombin (1U ml^{-1}), bradykinin (1nM , 10nM) or ATP ($0.3\mu\text{M}$) resulted in a fall in $[\text{Ca}^{2+}]_i$. These findings suggest that activation of protein kinase C inhibits mobilisation of calcium in BAEC.

10. The inhibitory effects of PMA (100nM) were attenuated by staurosporine (100nM), an inhibitor of protein kinase C, but not mimicked by the inactive phorbol ester, 4α -phorbol 12,13 didecanoate (4α -PDD; 100nM). Furthermore, staurosporine (100nM) increased $[\text{Ca}^{2+}]_i$ when added during the plateau phase of the increase in $[\text{Ca}^{2+}]_i$ induced by bradykinin (10nM) or thrombin (1U ml^{-1}). In contrast, staurosporine (100nM) reduced $[\text{Ca}^{2+}]_i$ when added during the plateau phase of the increase in $[\text{Ca}^{2+}]_i$ induced by ATP ($30\mu\text{M}$). These findings suggest that PMA may exert its actions via the activation of protein kinase C and that agonist-induced protein kinase C activation may modulate $[\text{Ca}^{2+}]_i$ in BAEC.

11. The ability of manganese (Mn^{2+}) to quench the fluorescence of fura-2 was used as a marker for bivalent cation entry into endothelial cells. Using this technique,

PMA (100nM), but not 4 -PDD (100nM), was found to inhibit the thrombin-induced Mn^{2+} quench of fura-2, suggesting that PMA inhibits calcium entry into BAEC.

12. Addition of PMA (100nM) during the sustained elevation of $[Ca^{2+}]_i$ induced by a low concentration of ionomycin had no effect on $[Ca^{2+}]_i$, suggesting that activation of protein kinase C does not promote calcium efflux in BAEC.

13. An attempt was made to determine whether cyclic AMP modulates calcium mobilisation in BAEC. Pre-treatment with forskolin (10 μ M), an activator of adenylate cyclase, had no effect on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (1U ml⁻¹), bradykinin (1nM, 10nM) or ATP (30 μ M). Furthermore, Pre-treatment with isoprenaline (10 μ M) had no effect on the initial transient elevation of $[Ca^{2+}]_i$ induced by bradykinin (3nM). In contrast, forskolin (10 μ M) and isoprenaline (10 μ M) each induced biphasic elevations of $[Ca^{2+}]_i$ when added during the plateau phase of the increase in $[Ca^{2+}]_i$, induced by the three agonists. In the presence of the inhibitor of calcium influx, nickel chloride (4mM), these biphasic elevations were reduced to monophasic transient elevations.

14. Pre-treatment with forskolin (10 μ M) had no effect on the ability of PMA (100nM) to attenuate the thrombin-induced plateau phase of $[Ca^{2+}]_i$, suggesting that forskolin does not enhance $[Ca^{2+}]_i$ by inhibiting the actions of protein kinase C.

15. 8 bromo cyclic GMP (30 μ M), a membrane-permeant analogue of cyclic GMP, had no effect on basal $[Ca^{2+}]_i$ or on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (1U ml⁻¹), bradykinin (10nM) or ATP (30 μ M). Furthermore, 8 bromo cyclic GMP (30 μ M) and sodium nitroprusside (1 μ M), an activator of soluble guanylate cyclase, had no effect when added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by each of the three agonists. These findings suggest that cyclic GMP

does not modulate calcium mobilisation in BAEC.

16. N^{G} -nitro-L-arginine ($50\mu\text{M}$), an inhibitor of nitric oxide synthase, had no effect on the magnitude of the initial transient elevation of $[\text{Ca}^{2+}]_{\text{i}}$ induced by thrombin (1U ml^{-1}), bradykinin (1nM) or ATP ($3\mu\text{M}$), and had no effect on the plateau phase of the increase in $[\text{Ca}^{2+}]_{\text{i}}$ induced by these agents. These findings suggest that EDRF, via endogenously generated cyclic GMP, does not modulate calcium mobilisation in BAEC.

17. Pre-treatment with pertussis toxin (25ng ml^{-1} ; 20hrs) had no effect on mobilisation of $[\text{Ca}^{2+}]_{\text{i}}$ induced by thrombin (1U ml^{-1}), bradykinin (10nM) or ATP ($30\mu\text{M}$), suggesting that the G proteins involved in the regulation of calcium mobilisation in BAEC are pertussis toxin-insensitive.

18. These findings suggest that, in bovine aortic endothelial cells, the agonist-induced transient elevation of $[\text{Ca}^{2+}]_{\text{i}}$ is completely dependent upon release of calcium from intracellular stores and the sustained component is due to calcium influx. The intracellular calcium stores may consist of two pools, one of which is rapidly depleted in the absence of extracellular calcium and a second which is resistant to such depletion.

Furthermore, these findings suggest that activation of protein kinase C inhibits, elevation of cyclic AMP content augments and elevation of cyclic GMP has no effect on calcium mobilisation in bovine aortic endothelial cells.

B. Role of calcium mobilisation in the regulation of endothelial barrier function

1. Cultures of bovine pulmonary artery endothelial cells (BPAEC) were seeded and grown to confluence on Transwell membrane filters. These endothelial monolayers restricted

the passage of trypan blue-labelled albumin and, thus, mimicked the in vivo endothelial barrier.

2. Albumin transfer across monolayers of BPAEC was increased by both thrombin (1U ml^{-1} ; 90min) and phorbol myristate acetate (PMA; 600nM; 90min), an activator of protein kinase C.

3. It has been proposed that elevation of cyclic AMP content enhances endothelial barrier function in vivo. Co-incubation with forskolin ($30\mu\text{M}$; 90min), an activator of adenylate cyclase, abolished both the thrombin (1U ml^{-1}) and PMA (600nM)-induced increases in albumin transfer across monolayers of BPAEC, suggesting that elevation of cyclic AMP enhances endothelial barrier function.

4. An attempt was made to determine whether elevation of cyclic GMP content could modulate endothelial barrier function. Co-incubation with atriopeptin II (100nM; 90min), an activator of particulate guanylate cyclase, or 8 bromo cyclic GMP ($30\mu\text{M}$), a membrane permeant analogue of cyclic GMP, had no effect on PMA (600nM)-induced albumin transfer, but abolished that induced by thrombin (1U ml^{-1}). These findings suggest that cyclic GMP enhances endothelial barrier function.

5. Pre-treatment of BPAEC with PMA (100nM, 5min) had no effect on basal $[\text{Ca}^{2+}]_i$, but inhibited thrombin (1U ml^{-1})-induced calcium mobilisation. These findings suggest that elevations of $[\text{Ca}^{2+}]_i$ are not a pre-requisite for increased albumin transfer and that activation of protein kinase C may regulate receptor-mediated processes in these cells.

6. An attempt was made to determine whether elevation of cyclic GMP content inhibits thrombin-induced albumin transfer via the inhibition of calcium mobilisation. However, pre-treatment with forskolin ($30\mu\text{M}$; 5min) had no effect on the magnitude of the thrombin (1U ml^{-1})-induced initial transient elevation of $[\text{Ca}^{2+}]_i$. In contrast,

addition of forskolin ($30\mu\text{M}$), during the plateau phase of the increase in $[\text{Ca}^{2+}]_i$ induced by thrombin (1U ml^{-1}), increased $[\text{Ca}^{2+}]_i$. These findings suggest that cyclic AMP does not inhibit thrombin-induced albumin transfer by inhibiting calcium mobilisation.

7. Pre-treatment of BPAEC with atriopeptin II (100nM , 5 min) or 8 bromo cyclic GMP ($30\mu\text{M}$, 5 min) had no effect on the magnitude of the initial transient elevation of $[\text{Ca}^{2+}]_i$ induced by thrombin (1U ml^{-1}). In contrast, addition of atriopeptin II (100nM) or 8 bromo cyclic GMP ($30\mu\text{M}$) during the thrombin-induced plateau phase of $[\text{Ca}^{2+}]_i$ resulted in a small increase in $[\text{Ca}^{2+}]_i$. These findings suggest that cyclic GMP does not inhibit thrombin-induced albumin transfer via the inhibition of calcium mobilisation.

9. These findings suggest that elevation of cyclic AMP or cyclic AMP content enhances endothelial barrier function. Cyclic AMP may inhibit the actions of protein kinase C, while cyclic GMP may inhibit a step proximal to the activation of protein kinase C.

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ABBREVIATIONS

ADP	Adenosine diphosphate
AM	Acetoxymethylester
AMP	5'-adenosine monophosphate
ANF	Atrial natriuretic factors
APII	Atriopeptin II
ATP	Adenosine triphosphate
BAEC	Bovine aortic endothelial cells
BK	Bradykinin
BPAEC	Bovine pulmonary artery endothelial cells
8BrcGMP	8 bromo guanosine - 3':5' cyclic monophosphate
BSA	Bovine serum albumin
[Ca ²⁺]	Calcium concentration
[Ca ²⁺] _i	Concentration of intracellular free calcium
CGRP	Calcitonin gene-related peptide
CO ₂	Carbon dioxide
Cyclic AMP/cAMP	Adenosine - 3':5' cyclic monophosphate
Cyclic GMP/cGMP	Guanosine - 3':5' cyclic monophosphate
DAG	Diacylglycerol
DMEM	Dulbecco's modification of Eagle's medium
DMSO	Dimethylsulphoxide
EC ₅₀	Concentration yielding 50% of maximum response
ECAM	Endothelial cell-adhesion molecule
EDHF	Endothelium-derived hyperpolarising factor
EDRF	Endothelium-derived relaxing factor
EDTA	Ethylenediamine tetra-acetate
EGTA	Ethylene glycol (β-aminoethylethane) N,N,N',N'-tetracetic acid
ET	Endothelin
FOR	Forskolin

GMP	5'-guanosine monophosphate
G protein	Guanine nucleotide-dependent regulatory protein
GTP	Guanosine triphosphate
HIST	Histamine
ICAM	Inducible cell-adhesion molecule
Ins(1,4,5)P ₃	Inositol 1,4,5 trisphosphate
Ins(1,3,4,5)P ₄	Inositol 1,3,4,5 tetrakisphosphate
IONO	Ionomycin
ISO	Isoprenaline
K _d	Dissociation constant
kDa	Kilodalton
6 Keto-PGF ₁ α	6 Keto-prostaglandin F ₁ α
L-NAME	N ^G -nitro-L-arginine methyl ester
LDL	Low density lipoprotein
L-NMMA	N ^G -monomethyl-L-arginine
L-NOARG	N ^G -nitro-L-arginine
LPS	Lipopolysaccharide
min	Minutes
MLCK	Myosin light-chain kinase
mRNA	Messenger RNA
NBCS	New born calf serum
PAF	Platelet activating factor
4α-PDD	4α-phorbol 12, 13 didecanoate
PGI ₂	Prostacyclin
PIC	Phosphoinositidase C
PMA	4β-phorbol 12-myristate 13-acetate
PROC	Procaine
PT	Pertussis toxin
PtdIns	Phosphatidylinositol
PtdIns(4)P	Phosphatidylinositol-4-phosphate
PtdIns(4,5)P ₂	Phosphatidylinositol-4,5-bisphosphate
R	Ratio
R _{max}	Maximal ratio (340:380)
R _{min}	Minimal ratio (340:380)
ROC	Receptor operated channel
S _{b2}	Fluorescence emission measured at an excitation wavelength of 380nm in the presence of saturating calcium

S_{f2}	Fluorescence emission measured at an excitation wavelength of 380nm in the absence of calcium
SNP	Sodium nitroprusside
STSP	Staurosporine
THR	Thrombin
TMB-8	3,4,5-trimethoxybenzoic acid 8-diethyl-amino acetyl ester
TNF	Tumour necrosis factor
VOC	Voltage operated channel

INTRODUCTION

CHAPTER 1

1.1 Role of the endothelium in the regulation of haemostasis and vasomotor tone

The vascular endothelium, which consists of a single layer of cells resting on a basement membrane in close apposition to the blood vessel wall, forms an interface between the blood and vascular smooth muscle and is, therefore, ideally positioned to modulate the functions of both components. The endothelium exerts many of its actions by secreting a number of physiologically important molecules. For example, vasomotor tone may be modulated by prostacyclin (Moncada et al., 1976a) or endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980), which may be nitric oxide (Palmer et al., 1987) or a nitric oxide-containing substance (Myers et al., 1990). The abluminal release of these substances in response to a number of physical and chemical stimuli (e.g. shear stress, acetylcholine) results in vasodilation. Release of EDRF may be of importance in the regulation of blood flow in vascular beds; for example, the coronary circulation (Amezcuca et al., 1988). The actions of EDRF and prostacyclin may be counter-balanced by the release of the endothelins, a class of vasoconstrictor peptides recently described by Yanagisawa et al. (1988). It is likely that accurate, physiological regulation of blood flow requires a precise balance between these antagonistic systems.

Luminal release of endothelium-derived substances is known to modulate haemostasis. The endothelium may inhibit coagulation by the production both of anticoagulant and fibrinolytic substances; for example, via production of thrombomodulin, an endothelial cell surface protein which binds thrombin and enhances its ability to activate protein C. Fibrinolysis may be promoted via two types of plasminogen activator, tissue plasminogen activator and urokinase type plasminogen activator (Levin & Loskutoff, 1982). However, under certain conditions, the endothelium may express procoagulant activity; for example, thrombin and endotoxin may induce production of thromboplastin (Brox et al., 1984). Coagulation may also be accelerated by endothelial production of factor V (Cervený et al., 1984) and plasminogen activator (Hanss & Collen, 1987).

The endothelium is normally non-thrombogenic, presumably due to the cell surface and to the release of EDRF and prostacyclin which inhibit platelet aggregation (Moncada et al., 1976a, b; Azuma et al., 1986; Furlong et al., 1987; Radomski et al., 1987a, b; Bhardwaj et al., 1988; Hogan et al., 1988). EDRF also inhibits platelet adhesion to the endothelium (Radomski et al., 1987c; Sneddon & Vane, 1988), although prostacyclin only inhibits the adhesion of activated platelets (Fry et al., 1980). Under different circumstances, such as during tissue injury, the endothelium may also promote platelet adhesion and aggregation via the production of collagen (Sage et al., 1979), thrombospondin (Mosher et al., 1982), fibronectin

(Jaffe & Mosher, 1978) and von Willebrand factor (Jaffe et al., 1973).

The ability of both endogenous and exogenous agents to control vasomotor tone and haemostasis may be regulated by endothelial cell metabolism. For example, the endothelial membrane contains angiotensin-converting enzyme, a carboxypeptidase which may regulate vasomotor tone by inactivating bradykinin, a potent vasodilator, while converting angiotensin I to angiotensin II, a potent vasoconstrictor (Fishman et al., 1974). The metabolism of adenine nucleotides (Pearson et al., 1980) may completely alter the responses produced by these mediators. For example, the ability of ADP, secreted from aggregating platelets, to induce further aggregation, may be attenuated by the metabolism of ADP to adenosine, an inhibitor of platelet aggregation.

1.2 Endothelium-derived relaxing factor (EDRF)

1.2.1 Discovery of EDRF

Furchgott and Zawadzki (1980) discovered that the relaxant actions of acetylcholine were dependent upon the release of a non-prostanoid relaxant, which was termed endothelium-derived relaxing factor (EDRF). A wide variety of stimuli has been shown to induce EDRF release, including bradykinin, ATP, thrombin, histamine, substance P and the calcium ionophore A23187 (Furchgott, 1984).

The first bioassay for EDRF was developed by Furchgott and Zawadzki (1980) who used a sandwich-mount preparation of both an endothelium-denuded and an endothelium-intact rabbit aortic strip. Addition of acetylcholine to this preparation resulted in the relaxation of the endothelium-denuded strip, demonstrating that EDRF is a diffusible substance. Griffith et al., (1984) determined the half-life of EDRF by examining the dilation of a pre-constricted rabbit coronary artery by the effluent from an acetylcholine-stimulated rabbit aorta. By increasing the transit time of the effluent to the sensor, a half-life of 6 seconds was calculated for EDRF. Similar values (6-49s) have been obtained in other studies (Förstermann et al., 1984; Cocks et al., 1985; Rubanyi et al., 1985; Gryglewski et al., 1986).

1.2.2 Nature of EDRF

Much speculation has been generated concerning the nature of EDRF. However, it now appears that EDRF may be nitric oxide, or a closely related derivative. Support for this proposal is provided by a strong line of evidence. For example, the relaxation induced by both nitric oxide and EDRF is associated with increased smooth muscle cyclic GMP content (Greutter et al., 1981; Rapoport & Murad, 1983). Furthermore, nitric oxide and EDRF activity is potentiated by superoxide dismutase (Gryglewski et al., 1986) and inhibited by haemoglobin (Martin et al., 1985; Furchgott et al., 1987). Using a chemiluminescence technique, Palmer et al. (1987) have demonstrated that bradykinin induces the

concentration-dependent release of both EDRF and nitric oxide from cultured endothelial cells. This latter point has been disputed by Myers and co-workers (1989) who claim that the amount of EDRF released is not sufficient to account for relaxation induced by EDRF. Furthermore, differences in the comparative pharmacology of EDRF and nitric oxide have been described. EDRF has been proposed to preferentially relax vascular smooth muscle, while nitric oxide has a more widespread action (Shikano et al., 1988). A difference in the chemical nature of these relaxants has been proposed by Cocks et al. (1988), who have shown that EDRF is negatively charged, while nitric oxide is a polar molecule. It is, therefore, possible that the nitric oxide released from the endothelium is derived from a large molecule, which has a stabilising role. As the incorporation of nitric oxide into a nitrosothiol group increases the potency and stability of this molecule, Myers et al. (1990) have proposed that S-nitrosocysteine or a similar substance may mimic the actions of EDRF more closely. However, the release of this compound has yet to be demonstrated.

1.2.3 Synthesis of nitric oxide

The vascular endothelial cell synthesizes nitric oxide from the terminal guanidino nitrogen of L-arginine by the action of nitric oxide synthase (Palmer et al., 1988a, b; Schmidt et al., 1988), the co-product of this reaction being L-citrulline which lacks vasodilating activity. The ability of N^G-monomethyl-L-arginine (L-NMMA) to inhibit the

enzymatic production of nitric oxide and citrulline (Palmer & Moncada, 1989), due to the presence of a methyl group on the guanidino nitrogen in L-NMMA, and the reversibility of the inhibitory actions of L-NMMA by L-arginine, suggests that L-arginine is likely to be the physiological precursor for the production of nitric oxide/EDRF. Nitric oxide appears to be produced in a diverse range of cell types, in addition to the endothelium. For example, in smooth muscle, platelets, neutrophils, macrophages, liver and neurones (Wood et al., 1990; Radomski et al., 1990; Rimele et al., 1988; Hibbs et al., 1988; Knowles et al., 1990; Gillespie et al., 1990; Garthwaite et al., 1988). In some tissues, such as the endothelium and the brain, nitric oxide synthase is constitutively expressed and calcium-calmodulin-dependent (Palmer et al., 1989; Mayer et al., 1989; Busse & Mülsch, 1990; Förstermann et al., 1990; Knowles et al., 1990). However, in other cells, including smooth muscle, hepatocytes and macrophages, the enzyme is not normally present, but may be induced by endotoxin (Stuehr & Marletta, 1985; Knowles et al., 1990). This inducible form of the enzyme is calcium-independent in some tissues (e.g. macrophages), but calcium-dependent in others (e.g. neutrophils) (Hiki et al., 1991).

1.2.4 Actions of EDRF in vascular smooth muscle

EDRF and the nitrovasodilators both relax smooth muscle and elevate cyclic GMP content (Katsuki et al., 1977; Rapoport & Murad, 1983), with the elevation of cyclic GMP content occurring prior to muscle relaxation. This observation,

coupled with the relaxation of smooth muscle induced by 8 bromo cyclic GMP, a membrane-permeant analogue of cyclic GMP (Collins et al., 1986), suggests increases in cyclic GMP levels are closely associated with relaxation. Further evidence is provided by the ability of haemoglobin, which binds EDRF, and methylene blue, which oxidizes the iron in the haem prosthetic group from the ferrous to the ferric oxidation state, to inhibit the ability of EDRF to induce relaxation and increase cyclic GMP (Martin et al., 1985; Griffith et al., 1985). Zaprinst, an inhibitor of cyclic GMP phosphodiesterase, potentiates the actions of EDRF, and further supports a role for cyclic GMP. Rapoport et al. (1983) demonstrated that the changes in cyclic GMP induced by EDRF were associated with increased protein phosphorylation, suggesting that EDRF may alter the activity of key cellular proteins.

Smooth muscle contraction is dependent upon calcium-calmodulin-dependent phosphorylation of myosin light-chains, via the enzyme myosin light-chain kinase (MLCK). However, in contrast to the ability of cyclic AMP-dependent protein kinase to inhibit the calcium sensitivity of purified MLCK, the enzyme activity is unaffected by cyclic GMP-dependent protein kinase (Hathaway et al., 1985). In contrast, Itoh et al. (1985) have shown that cyclic GMP alters the calcium sensitivity of skinned muscle fibres, suggesting that a key regulatory component may be absent from the purified MLCK.

Cyclic GMP is thought to produce its actions predominantly through the inhibition of calcium mobilisation (Collins et al., 1986) and promotion of calcium efflux (Rashatwar et al., 1987). It has been suggested that cyclic GMP inhibits calcium influx through receptor operated channels (ROCs) to a greater degree than calcium influx through voltage operated channels (VOCs) (Collins et al., 1988). Inhibition of calcium influx through ROCs may result, in part, from the cyclic GMP-mediated inhibition of phosphoinositide hydrolysis (Rapoport, 1986). Hence, as smooth muscle contraction is dependent on calcium-calmodulin-induced activation of MLCK, the lowering of cytosolic calcium will result in relaxation.

1.2.5 Actions of EDRF in platelets

EDRF is a potent inhibitor of platelet activation. It has been shown to inhibit aggregation in vitro (Azuma et al., 1986; Furlong et al., 1987) and in vivo (Bhardwaj et al., 1988; Hogan et al., 1988) and to block platelet adhesion to the vascular endothelium (Radomski et al., 1987c; Sneddon & Vane, 1988). These luminal actions of EDRF may have a key role in the regulation of haemostasis. More recently, Radomski et al. (1990) have suggested that platelets themselves may contain an endogenous nitric oxide-L-arginine system and that this may have a negative feedback role in the regulation of platelet function. The inhibitory actions of EDRF are likely to reflect the ability of cyclic GMP to inhibit platelet secretion, phosphoinositide hydrolysis and calcium mobilisation (Takai

et al., 1981; MacIntyre et al., 1985b). The inhibitory effects of EDRF on platelet aggregation are potentiated by prostacyclin (Radomski et al., 1987b) and, therefore, the basal release of both substances may be sufficient to exert a tonic inhibitory action.

1.3 Prostacyclin

1.3.1 Discovery and synthesis of prostacyclin

Prostacyclin, or PGI_2 , originally termed PGX, was discovered in 1976 and found to be an unstable substance, produced by enzymes isolated from blood vessel walls, which inhibited platelet aggregation (Moncada et al., 1976a, b). Prostacyclin production depends upon the liberation of arachidonic acid from membrane phospholipids by phospholipase A_2 . This is the calcium-dependent step in the production of prostacyclin (Van Den Bosch, 1980). Arachidonic acid is then converted to prostaglandin G_2 by cyclo-oxygenase, an enzyme which cyclizes the arachidonate and, subsequently, adds a 15-hydroperoxy functional group. Prostaglandin G_2 is converted to prostaglandin H_2 via a peroxidase reaction and the subsequent isomerization of prostaglandin H_2 , by prostacyclin synthase, yields prostacyclin. The half-life of prostacyclin at physiological pH is 3 minutes and longer than that of EDRF. Prostacyclin is mainly broken down by a non-enzymic reaction to the inactive product, 6-keto-PGF $_{1\alpha}$. Cultured vascular endothelial cells and perfused tissues release prostacyclin in response to a number of stimuli, including

thrombin, trypsin, A23187, ATP, histamine, leukotriene C₄, leukotriene D₄ and bradykinin (Weksler et al., 1978; Pearson et al., 1983; Johnson et al., 1985; Clark et al., 1986), suggesting that this prostanoid may play an important role in the regulation of vasomotor tone.

1.3.2 Actions of prostacyclin in vascular smooth muscle

Prostacyclin exerts its physiological actions through binding to a membrane-associated receptor, termed the IP-receptor. Prostacyclin is a potent vasodilator (Armstrong et al., 1978) and this action is associated with stimulation of adenylate cyclase and elevated cyclic AMP content in vascular smooth muscle (Dembinska - Kiec et al., 1980).

Cyclic AMP appears to induce relaxation by a number of mechanisms, all via phosphorylation by cyclic AMP-dependent protein kinase. For example, the phosphorylation of myosin light-chain kinase (MLCK) by cyclic AMP-dependent protein kinase (Conti & Adelstein, 1981; Hathaway et al., 1985) increases the calcium-calmodulin requirement for activation of MLCK. Consequently, at a given level of cytosolic calcium, MLCK-induced phosphorylation of myosin light-chains will be reduced. An important additional mechanism inducing relaxation is the lowering of cytosolic calcium levels. Cyclic AMP inhibits calcium influx through VOCs in smooth muscle (Meisheri & van Breemen, 1982). Furthermore, the inhibition of noradrenaline-induced contractions in rat aorta, by elevation of cyclic AMP

content, suggests that calcium influx through ROCs may also be inhibited (Lincoln & Simpson, 1984). Elevation of cyclic AMP content inhibits the contraction induced by intracellular calcium release (van Eldere et al., 1982), presumably by attenuation of receptor-mediated phosphoinositide hydrolysis (Hall et al., 1989). Additionally, cytoplasmic calcium levels may be lowered via enhanced uptake into intracellular calcium stores (Itoh et al., 1985), a process which may involve the endoplasmic reticulum protein, phospholamban (Raeymaekers & Jones, 1986). In contrast, cyclic AMP does not appear to promote calcium extrusion in vascular smooth muscle (Rashatwar et al., 1987). Cyclic AMP has been reported to interact synergistically with cyclic GMP to relax smooth muscle (Grace et al., 1988), providing the possibility that prostacyclin and EDRF may potentiate the actions of each other in the vasculature.

1.3.3 Actions of prostacyclin in platelets

Prostacyclin-mediated inhibition of platelet aggregation is associated with increased cyclic AMP content (Tateson et al., 1977). Such elevations of platelet cyclic AMP content have been found to inhibit calcium mobilisation, phosphoinositide breakdown, aggregation, shape change and secretion (Takai et al., 1982; Sage & Rink, 1985). The ability of cyclic AMP and cyclic GMP to synergistically inhibit platelet phosphoinositide hydrolysis and secretion (Takai et al., 1982) is likely to explain the potent, synergistic inhibition of platelet function observed with

low concentrations of EDRF and prostacyclin (Radomski et al., 1987b).

1.4 **Endothelial ion fluxes : regulation of vasomotor tone**

1.4.1 Endothelial cell hyperpolarisation and endothelium-derived hyperpolarising factor (EDHF)

It is well documented that several agents which stimulate EDRF production, including ATP, bradykinin and A23187 also increase the efflux of $^{86}\text{Rb}^+$, a marker for potassium, in vascular endothelial cells (Gordon & Martin, 1983a). This increased $^{86}\text{Rb}^+$ efflux reflects the activation of calcium-activated potassium channels (Colden-Stanfield et al., 1987; Sauve et al., 1988). Endothelial hyperpolarisation, resulting from the activation of these potassium channels, has also been reported following stimulation with acetylcholine (Olesen et al., 1988b) or haemodynamic shear stress (Olesen et al., 1988a) and is thought to be functionally important. For example, smooth muscle relaxation may be mediated via the gap-junctional coupling of endothelial cells to smooth muscle cells (Davies, 1986). Although electronic spread of endothelial hyperpolarisation may explain the ability of muscarinic agonists to induce smooth muscle hyperpolarisation (Bolton et al., 1984), an alternative proposal involves the release of an unidentified substance, termed endothelium-derived hyperpolarising factor (EDHF) (Chen et al., 1988; Huang et al., 1988a). The differential release of two distinct

substances EDRF and EDHF is supported by the observations of Komori and Suzuki (1987) who demonstrated that activation of M_1 and M_2 receptors was required for production of EDHF and EDRF, respectively. In support of these proposals, haemoglobin, which binds and inactivates EDRF (Martin et al., 1985), was found to inhibit both acetylcholine-mediated relaxation in rat aorta and the associated elevation of cyclic GMP content, but had no effect on the hyperpolarisation produced (Chen et al., 1988). This supports the proposal that EDRF or nitric oxide does not mediate the hyperpolarisation. However, a recent study has demonstrated that nitric oxide can hyperpolarise vascular smooth muscle (Tare et al., 1990). Hence, EDRF may mediate some of its actions through hyperpolarisation. EDHF may exert a greater action on muscle membrane potential in some vascular beds, although it is not clear if this is a releasable factor or if it simply results from the electrotonic spread of current following receptor - or calcium - induced activation of potassium channels.

1.4.2 Regulation of endothelial function by mechanical stimulation of ion channels or by depolarisation

A study undertaken by Lansman et al. (1987) initially demonstrated the ability of mechanical stretch, presumably acting via mechanotransducers, to activate cation-selective channels in vascular endothelial cells. As EDRF and prostacyclin production is calcium dependent (Singer & Peach, 1982; Weksler et al., 1978), the increased entry of

calcium may serve to enhance release of these two vasoactive substances, leading to vasodilation. This proposal has been supported by direct measurement of cytosolic calcium levels using fura-2 demonstrating that mechanical stimulation elevates $[Ca^{2+}]_i$ by mobilisation of calcium from both intracellular and extracellular stores (Goligorsky, 1988).

In addition to the release of a vasoactive substance from the endothelium by mechanical stimulation, endothelial membrane depolarisation has been found to reduce $[Ca^{2+}]_i$ in vascular smooth muscle, via the production of a diffusible substance (Goligorsky, 1988). However, it is not clear how depolarisation promotes the release of this substance as endothelial cells do not possess voltage-operated calcium channels (Hallam & Pearson, 1986) and, in fact, agonist-induced EDRF production is actually inhibited by membrane depolarisation (Lückhoff & Busse, 1990).

1.5 **Endothelium-derived constricting factors**

1.5.1 The endothelins

The proposal that endothelial cells release a vasoconstrictor peptide (Hickey et al., 1985; Gillespie et al., 1986) was confirmed following the isolation of endothelin (now termed endothelin-1) from the culture supernatant of pig aortic endothelial cells (Yanagisawa et al., 1988). Endothelin-1, a 21 amino acid peptide, is synthesized from a 203 amino acid precursor by a two step

process: the precursor is first cleaved proteolytically, to produce big endothelin-1 (38 or 39 amino acids), and this is subsequently cleaved by an endoproteolytic process to yield endothelin-1 (Yanagisawa et al., 1988). This biological activity of endothelin-1 is 100 times greater than that of big endothelin (Yanagisawa & Masaki, 1989).

Endothelin-1 release is induced by a wide range of stimuli, including thrombin, A23187, angiotensin II, transforming growth factor β , interleukin-1, vasopressin and hypoxia (Yanagisawa et al., 1988; Emori et al., 1989; Yoshizumi et al., 1990; Hexum et al., 1990; Hieda et al., 1990). Although enhancement of endothelin-1 production is a relatively slow process, occurring over several hours and requiring increased mRNA production (Yanagisawa et al., 1988), shear stress has been proposed to induce rapid release of endothelin-1, within 3 minutes (Milner et al., 1990).

It is now known that there is a family of endothelin-like peptides consisting of endothelins-1, -2, -3 (ET-1, ET-2 and ET-3). Similar biological activity has been found in the sarafotoxins S6, a group of peptide toxins from the venom of the burrowing asp, *Atractaspis engaddensis*, suggesting a common evolutionary origin of these peptides. Three genes for endothelin have been cloned (Inoue et al., 1989) which separately encode for ET-1, ET-2 and ET-3, although only ET-1 has been detected in endothelial cells.

1.5.2 Actions of endothelins in vascular smooth muscle

Endothelin-1 induces large, sustained contractions of vascular smooth muscle in a number of blood vessels and vascular beds (for review, Yanagisawa & Masaki, 1989). Although ET-1 has a half life of only 2 minutes and is rapidly eliminated from the circulation, the pressor response is much longer lasting.

Endothelin-1 stimulates phosphoinositidase C-induced hydrolysis of phosphatidylinositol-4, 5-bisphosphate, which results in the generation of inositol (1,4,5) trisphosphate, calcium mobilisation and activation of protein kinase C via diacylglycerol (Resink et al., 1988; Marsden et al., 1989; Griendling et al., 1989). The activation of protein kinase C by diacylglycerol may account for the sustained contraction, as activation of protein kinase C, via phorbol esters, induces a slow, long lasting contraction (Itoh et al., 1988) similar to that obtained with endothelin-1. Diacylglycerol accumulation may also occur as a result of phosphatidylcholine hydrolysis by endothelin (MacNulty et al., 1990). Originally, endothelin-1 was proposed to directly activate a dihydropyridine-sensitive calcium channel (Yanagisawa et al., 1988), although van Renterghem and co-workers (1988) have suggested that these calcium channels are opened subsequent to membrane depolarisation.

In addition to powerful vasoconstricting activity, certain endothelins also have vasorelaxant activity. Endothelin-1

and endothelin-3 induce release of EDRF and prostacyclin from perfused vascular beds (De Nucci et al., 1988b; Warner et al., 1989) and this may occur as a result of endothelial phosphoinositide breakdown and calcium mobilisation (Emori et al., 1990, 1991).

The discovery of 3 distinct endothelin peptides, as well as a snake venom counterpart, suggests the possible existence of receptor subtypes. In support of this, 2 distinct endothelin receptors have recently been described, in cardiac membranes, on the basis of differing agonist affinities (Watanabe et al., 1989).

1.5.3 Other endothelium-derived constricting factors

In addition to the endothelins, other substances may be involved in the production of rapid, endothelium-dependent contractions. For example, an arachidonic acid metabolite may be involved in the production of endothelium-dependent contractions induced by agents such as acetylcholine or serotonin (Miller & Vanhoutte, 1985; Lüscher & Vanhoutte, 1986 a, b). Release of this factor during hypoxia may contribute to hypoxia-induced vasospasm (Vanhoutte, 1988).

An alternative constricting factor may be superoxide anion. The endothelium can produce large quantities of superoxide anion (Matsubara & Ziff, 1986), but its origin is unclear. It may be produced, in part, by endothelial cyclo-oxygenase activation, as a consequence of arachidonate metabolism (Kontos et al., 1985). The contractile actions of

superoxide anion are likely to be explained by its ability to inactivate EDRF (Rubyani & Vanhoutte, 1986; Gryglewski et al., 1986).

1.6 Calcium and inositol phosphates

1.6.1 The phosphatidylinositol pathway

The first indication that phospholipid metabolism was involved in cellular signalling was demonstrated by Hokin and Hokin (1953), who observed that acetylcholine could enhance phospholipid metabolism and, specifically, the incorporation of ^{32}P into phosphatidylinositol (PtdIns) and phosphatidic acid. Several years later, Michell (1975) suggested the potential importance of this signalling pathway by illustrating the correlation between PtdIns hydrolysis by phospholipase C and calcium mobilisation. Recent studies have shown that several types of phospholipase C exist, including a PtdIns specific-phospholipase C and phosphatidylcholine - specific phospholipase C. The former enzyme is now commonly termed phosphoinositidase C, and this term is used throughout the text. In most tissues, 80-85% of the inositol lipid is PtdIns, with variable, but similar, amounts of phosphatidylinositol-4-phosphate (PtdIns(4)P) and phosphatidylinositol-4, 5-bisphosphate (PtdIns(4,5)P₂). Sequential phosphorylation of PtdIns on the 4- and 5-hydroxyl groups of inositol yields PtdIns(4)P and PtdIns(4,5)P₂, respectively. It is now apparent that the main pathway for inositol lipid metabolism involves the

receptor-mediated hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ to yield inositol - 1,4,5 - trisphosphate ($\text{Ins}(1,4,5)\text{P}_3$) and diacylglycerol (DAG) (Michell et al., 1981). $\text{Ins}(1,4,5)\text{P}_3$ is the first metabolite to be produced upon receptor activation and this is followed by the production of inositol - 1,3,4,5 - tetrakisphosphate ($\text{Ins}(1,3,4,5)\text{P}_4$), inositol - 1,4 - bisphosphate (Wollheim & Biden, 1986). Subsequent dephosphorylation of the tris and tetrakisphosphate isomers yields inositol bisphosphates, inositol monophosphates and, finally, inositol which may then be re-utilized in the synthesis of PtdIns .

1.6.2 Mechanism of inositol phosphate-induced calcium mobilisation

The importance of calcium in the regulation of physiological function has long been appreciated since the observation made by Ringer (1883) that this ion was required to sustain contraction of cardiac muscle. Calcium exerts its action as an intracellular messenger by two mechanisms; it can bind directly to certain effector proteins, such as troponin C in skeletal muscle or, alternatively, it may first bind to the specific calcium binding protein, calmodulin (Cheung, 1980), which then activates specific protein kinases.

Although $\text{Ins}(1,4,5)\text{P}_3$ and, more speculatively, $\text{Ins}(1,3,4,5)\text{P}_4$ may have a major role in the regulation of calcium mobilisation (Streb et al., 1983; Berridge, 1984; Irvine & Moor, 1986, 1987), the precise location of their

intracellular sites of action is not entirely clear. $\text{Ins}(1,4,5)\text{P}_3$ induces release of calcium from non-mitochondrial intracellular calcium stores in permeabilized cells (Streb et al., 1983). Initial studies suggested that these calcium stores may reside in the endoplasmic reticulum (Streb et al., 1984). A major role for $\text{Ins}(1,4,5)\text{P}_3$ is supported by the observation that the injection of $\text{Ins}(1,4,5)\text{P}_3$ mimics the effects of receptor activation (Oron et al., 1985). However, recent studies have suggested that the site of $\text{Ins}(1,4,5)\text{P}_3$ action may not be the endoplasmic reticulum and the existence of a subcellular organelle, termed the "calciosome" has been proposed (Krause et al., 1989). $\text{Ins}(1,4,5)\text{P}_3$ exerts its actions through a specific receptor which has been cloned and its primary structure determined (Furuichi et al., 1989).

Production of $\text{Ins}(1,3,4,5)\text{P}_4$ occurs via the phosphorylation of $\text{Ins}(1,4,5)\text{P}_3$ by the calcium-dependent 3-kinase (Biden & Wollheim, 1986). Hence, $\text{Ins}(1,4,5)\text{P}_3$ -induced calcium mobilisation may promote the formation of $\text{Ins}(1,3,4,5)\text{P}_4$. It has also been proposed that the 3-kinase may be directly coupled to receptor activation in some cells (Irvine et al., 1988). It has been further suggested that the $\text{Ins}(1,4,5)\text{P}_3$ -induced release of calcium from intracellular pools may act in conjunction with $\text{Ins}(1,3,4,5)\text{P}_4$ to gate calcium entry at the plasma membrane (Irvine & Moor, 1986, 1987; Irvine et al., 1988). In this system, $\text{Ins}(1,3,4,5)\text{P}_4$ may have a permissive role in the action of

Ins(1,4,5)P₃ by controlling the state of the Ins(1,4,5)P₃-sensitive intracellular calcium stores, through regulation of calcium entry (Figure 1.1). Recent work suggests that Ins(1,3,4,5)P₄ may also regulate the movement of intracellular calcium from Ins(1,4,5)P₃-insensitive stores (Irvine et al., 1988). This Ins(1,3,4,5)P₄-regulated process may require the presence of GTP (Irvine et al., 1988), which has been previously shown to regulate the formation of gap junctions between membranes (Mullaney et al., 1987).

In a number of cells, calcium entry may also occur in the absence of inositol phosphate generation, influx being directly coupled to receptor activation. One well studied example is the ATP receptor-operated channel in vascular smooth muscle (Benham & Tsien, 1987).

When measuring agonist-induced elevations of [Ca²⁺]_i in a population of cells, using fluorescent indicators, a biphasic elevation of [Ca²⁺]_i is often observed. For example, in endothelial cells, platelets, smooth muscle and neutrophils (Rotrosen & Gallin, 1986; Sage & Rink, 1985; Itoh et al., 1988; McCarthy et al., 1989). However, single cell analysis of the changes in [Ca²⁺]_i (Jacob et al., 1988), shows that they consist of repetitive, large, transient elevations of [Ca²⁺]_i spiking from either resting levels of [Ca²⁺]_i or an elevated level of [Ca²⁺]_i. It is likely, therefore, that the biphasic elevations of [Ca²⁺]_i observed in populations of cells reflects the mean [Ca²⁺]_i

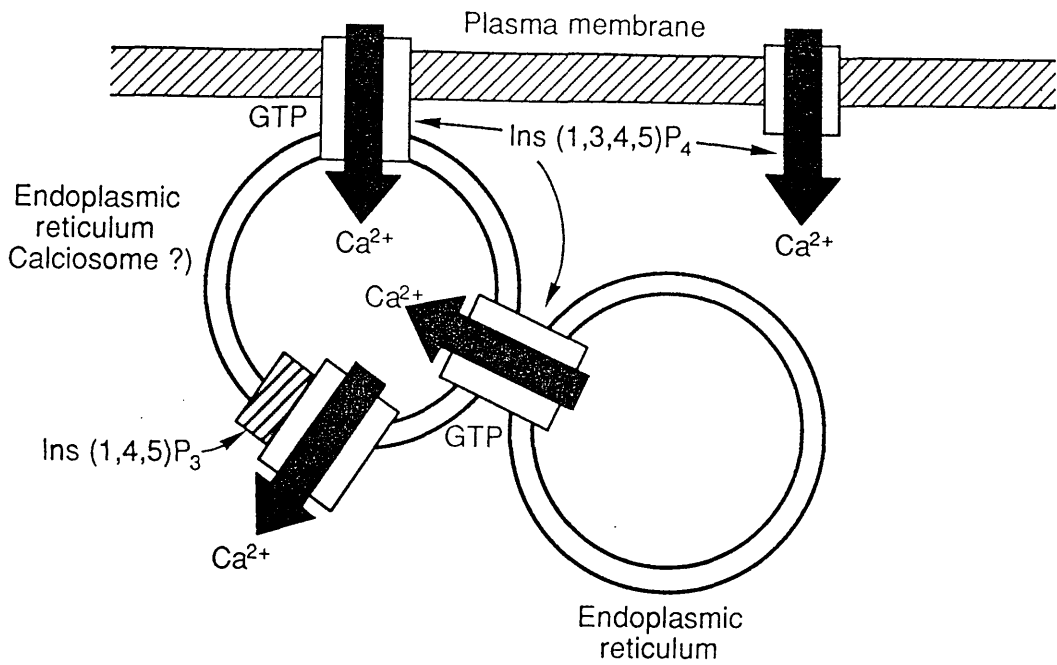


Figure 1.1 Schematic diagram of proposed actions of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 (reproduced and modified from Irvine et al., 1988). Ins(1,4,5)P_3 releases calcium from an intracellular store which may be the endoplasmic reticulum or a more specialised organelle, termed the calciosome. Ins(1,3,4,5)P_4 may induce calcium mobilisation by a number of mechanisms. These include (a) gating of calcium into the Ins(1,4,5)P_3 -sensitive store from the extracellular space, (b) transfer of calcium from the Ins(1,4,5)P_3 -insensitive store and (c) direct gating of calcium into the cytosol from the extracellular space. The actions of Ins(1,3,4,5)P_4 may require the presence of GTP.

signal from thousands of cells which are oscillating asynchronously.

1.6.3 Calcium oscillations

Measurement of $[Ca^{2+}]_i$ in cells which are electrically inexcitable led to the discovery that stimuli which activate PtdIns metabolism, often induce periodic oscillations of $[Ca^{2+}]_i$. This may allow the digital coding of cellular responses following exposure to external stimuli. Several theoretical models have been proposed to account for these oscillations in $[Ca^{2+}]_i$ and they all involve $Ins(1,4,5)P_3$, although they differ on whether or not levels of $Ins(1,4,5)P_3$ actually oscillate. These models have been sub-divided into those that are receptor-controlled and those that are second-messenger controlled (Berridge, 1990). With receptor-controlled models, potential feedback mechanisms may exert their actions at the level of receptor-mediated $Ins(1,4,5)P_3$ production, resulting in oscillations in $Ins(1,4,5)P_3$ levels. For example, protein kinase C could exert a negative feedback action on phosphoinositidase C activation by the uncoupling of receptor -G protein interactions (Woods et al., 1987). This would result in an inhibition of $Ins(1,4,5)P_3$ generation and calcium mobilisation until the actions of cellular phosphatases reverse the actions of protein kinase C, allowing a subsequent burst of $Ins(1,4,5)P_3$ generation and calcium mobilisation. An alternative mechanism has been proposed by Meyer and Stryer (1988), who suggest that $Ins(1,4,5)P_3$ -induced elevations of

$[Ca^{2+}]_i$ exert a positive feedback action on phosphoinositidase C, thus enhancing both $Ins(1,4,5)P_3$ production and calcium mobilisation. Depletion of the $Ins(1,4,5)P_3$ -sensitive store and the sequestration of calcium by other sites, results in a fall in $[Ca^{2+}]_i$ and the termination of the response. Therefore, refilling of the $Ins(1,4,5)P_3$ -sensitive calcium stores is required before the next burst of calcium release can be obtained despite the continued presence of $Ins(1,4,5)P_3$.

Second messenger-controlled models are dependent upon near constant levels of $Ins(1,4,5)P_3$. The model proposed by Gray (1988) suggests that elevated $[Ca^{2+}]_i$ inhibits the release of calcium from intracellular stores. Recent observations suggest that calcium may exert this negative feedback inhibition by acting at the $Ins(1,4,5)P_3$ receptor (Pietri et al., 1990; Payne et al., 1990). An alternative model depends upon the existence of two releasable stores of calcium, an $Ins(1,4,5)P_3$ -sensitive store and an $Ins(1,4,5)P_3$ -insensitive store (Berridge & Galione, 1988). $Ins(1,4,5)P_3$ induces calcium mobilisation and this calcium, along with calcium which enters the cytosol from the extracellular space, is taken up into $Ins(1,4,5)P_3$ -insensitive stores. This eventually results in overloading of these stores and the discharge of excess calcium into the cytosol occurs. The periodic loading and discharge of these stores is proposed to produce oscillations in $[Ca^{2+}]_i$.

1.6.4 Calcium, inositol phosphates and control of endothelial cell function

Direct measurement of calcium mobilisation, by the use of fluorescent calcium indicators, such as fura-2 or indo-1, and the measurement of inositol phosphate production, has strengthened the proposal that production of EDRF and prostacyclin is closely linked to the inositol phosphate/calcium system. For example, agents such as bradykinin, ATP, thrombin and histamine, which all increase EDRF and prostacyclin release, have been shown to elevate $[Ca^{2+}]_i$ (Lückhoff & Busse, 1986; Rotrosen & Gallin, 1986; Hallam & Pearson, 1986; Jaffe et al., 1987; Colden-Stanfield et al., 1987) and increase inositol phosphate production (Derian & Moskowitz, 1986; Piroton et al, 1987; Halldorsson et al., 1988) in the endothelium.

Calcium is an important regulator of endothelial cell function. Sustained release of EDRF is dependent upon the presence of extracellular calcium (Singer & Peach, 1982; Long & Stone, 1985; Lückhoff et al, 1988a; White & Martin, 1989). As described in section 1.2.2, EDRF is thought to be nitric oxide (Palmer et al., 1987) and this is produced in the endothelium by the enzyme, nitric oxide synthase. Activation of this enzyme occurs, concentration-dependently, within a calcium concentration range of 30-300nM (Mayer et al., 1989). As basal levels of $[Ca^{2+}]_i$ in endothelial cells are typically 60-100nM (Hallam & Pearson, 1986; Colden-Stanfield et al., 1987; Hallam et al., 1988a, b), nitric oxide synthase will be partially

activated, even in unstimulated cells; this may account for the observed basal release of EDRF (Griffith et al., 1984).

The calcium-induced activation of the constitutive nitric oxide synthase appears to be mediated through the calcium binding protein, calmodulin, rather than by a direct calcium-enzyme interaction (Förstermann et al., 1990; Büsse & Mülsch, 1990).

Calcium is also an important stimulus for the production of prostacyclin, as demonstrated by the ability of A23187 (a calcium ionophore) to induce release of prostacyclin from endothelial cell cultures (Weksler et al., 1978; Adams Brotherton & Hoak, 1982; Seid et al., 1983) and the ability of the calmodulin antagonist, W7, to inhibit its production (Seid et al., 1983). The calcium-dependent step is likely to be the activation of phospholipase A₂, the enzyme which produces arachidonic acid from membrane phospholipids (Van Den Bosch, 1980). Agonist-induced prostacyclin production is transient (Pearson et al., 1983; Gordon & Martin, 1983b) and this may reflect the dependence of prostacyclin production on the large, transient release of calcium from intracellular stores (Lückhoff et al., 1988a; Hallam et al., 1988a; White & Martin, 1989). This is supported by the ability of TMB-8, a putative intracellular calcium antagonist (Malagodi & Chiou, 1974), to inhibit endothelial prostacyclin production (Seid et al., 1983; Lückhoff et al., 1988a; White & Martin, 1989). Recent studies have suggested that elevation of $[Ca^{2+}]_i$ above a critical

threshold of approximately 800nM may be required for prostacyclin production, at least in human umbilical vein endothelial cells (Hallam et al., 1988a; Carter et al., 1989).

Calcium mobilisation has been shown to enhance endothelin production in the endothelium (Emori et al., 1989), although the enhanced production occurs over a period of hours, suggesting that calcium merely serves as a trigger in a complex sequence of events involving peptide synthesis.

1.7 Protein kinase C

1.7.1 Activation of protein kinase C

The ability of diacylglycerol (DAG) to promote activation of a protein kinase was first demonstrated by Nishizuka and co-workers (Takai et al., 1979; Kishimoto et al., 1980). This enzyme, termed protein kinase C, requires calcium and phospholipid, preferably phosphatidylserine, for its activation (Takai et al., 1979), and phosphorylates a wide range of substrates in many cell types (Nishizuka, 1986). The stimulatory actions of DAG on protein kinase C are mimicked by the tumour-promoting phorbol esters (Castagna et al., 1982), allowing these compounds to serve as useful pharmacological tools in the evaluation of the role of this enzyme, especially as they exert a prolonged stimulatory action on protein kinase C, in contrast to DAG which is rapidly metabolised.

The diacylglycerol required to activate protein kinase C may be derived from receptor-mediated hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ by phosphoinositidase C (Berridge, 1984). However, hydrolysis of this inositol lipid is known to be relatively transient and, therefore sustained production of DAG may derive from an alternative source, for example, phosphatidylinositol. Recent evidence has suggested that hydrolysis of phosphatidylcholine may be a useful alternative source of DAG in a wide range of tissues (Billah & Anthes, 1990). Phosphatidylcholine hydrolysis may be mediated by either a phospholipase C, which yields DAG and phosphocholine as products, or by a phospholipase D, which yields choline and phosphatidic acid. Phosphatidic acid may subsequently be catabolised to DAG by the enzyme phosphatidic acid phosphohydrolase.

1.7.2 Actions of protein kinase C

Protein kinase C has been described as a bidirectional regulator of cell function (Drummond, 1985). This reflects its ability to promote cellular activation (for example, hormone secretion, growth, contraction or metabolism), while simultaneously impairing the receptor-operated transduction processes by which receptors mediate these cellular functions. Hence, by phosphorylation of appropriate substrates, protein kinase C may promote positive responses, in addition to activating negative feedback regulatory pathways. This dual action has been demonstrated in the platelet where calcium mobilisation and protein kinase C activation synergistically promote

secretion (Rink et al., 1983) despite protein kinase C activation inhibiting inositol lipid metabolism and calcium mobilisation (MacIntyre et al., 1985a).

1.7.3 Protein kinase C and control of endothelial cell function

As with many other cell types, protein kinase C activation in endothelial cells may exert an inhibitory action on cell function or, alternatively, may promote cell activation. Agents, such as bradykinin, which enhance release of EDRF and prostacyclin, are known to generate diacylglycerol via hydrolysis of endothelial phosphoinositides (Derian & Moskowitz, 1986). This diacylglycerol may serve to regulate EDRF and prostacyclin production, as the tumour-promoting phorbol esters, which mimic the stimulatory action of diacylglycerol on protein kinase C (Castagna et al., 1982), inhibit endothelium-dependent relaxation induced by histamine in guinea-pig pulmonary artery (Weinheimer et al., 1986), acetylcholine and substance P in rabbit aorta (Lewis & Henderson, 1987) and acetylcholine in rabbit pulmonary artery (Cherry & Gillis, 1988). The inability of the phorbol esters to inhibit relaxations induced by nitroprusside (Lewis & Henderson, 1987), suggests that these compounds act by the inhibition of EDRF production and not by blockade of its actions. Furthermore, the inability of phorbol esters to suppress endothelium-dependent relaxations induced by the calcium ionophore, A23187, suggest that these compounds inhibit EDRF release via inhibition of receptor-mediated

mobilisation of calcium (Weinheimer et al., 1986; Lewis & Henderson, 1987; Cherry & Gillis, 1988). The phorbol ester-mediated inhibition of EDRF release has also been observed in studies using endothelial cell cultures (De Nucci et al., 1988a; Smith & Lang, 1990), illustrating the validity of using these cultured cell systems in the evaluation of endothelial cell function. However, in contrast to the above studies, Sakata and Karaki (1990) have recently demonstrated that phorbol esters may promote EDRF release from rat aortic rings. Hence, in addition to the well- documented negative feedback pathway, phorbol esters may also promote EDRF release.

Release of prostacyclin from bovine aortic endothelial cells can be induced either by phorbol esters or by increasing $[Ca^{2+}]_i$ (Demolle & Boeynaems, 1988). Simultaneous activation of both pathways results in a highly synergistic release of prostacyclin. Recent studies suggest that protein kinase C may enhance the calcium sensitivity of phospholipase A_2 , resulting in enhanced prostacyclin production at a given $[Ca^{2+}]_i$ (Carter et al., 1989). However, the actions of phorbol esters on prostacyclin production are complex, as demonstrated by the ability of these agents to inhibit thrombin-induced prostacyclin production, perhaps by inhibition of inositol phosphate production (Halldorsson et al., 1988).

Phorbol esters are known to promote release of endothelin, by a mechanism that is highly synergistic with elevations

of $[Ca^{2+}]_i$ (Emori et al., 1989). However, unlike the effects of phorbol esters on EDRF and prostacyclin, which occur within minutes, the effects of these agents on endothelin production occur over a number of hours.

1.8 Adenylate cyclase

1.8.1 Cyclic AMP

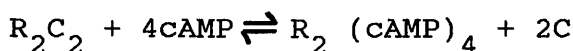
Cyclic AMP (adenosine - 3':5' cyclic monophosphate) is a nucleotide synthesised from adenosine triphosphate (ATP) by the action of adenylate cyclase, a transmembrane protein. A number of membrane-bound receptors are now known to exert their stimulatory actions on adenylate cyclase via guanine nucleotide regulatory proteins (G proteins) (Gilman, 1987). The G protein associated with stimulation of adenylate cyclase is termed G_s and is cholera toxin-sensitive. Stimulation of adenylate cyclase is thought to result from the direct interaction of the $G_{s\alpha}$ subunit with the enzyme, resulting in enhanced cyclic AMP turnover.

Activation of a number of receptors - for example, muscarinic receptors and α_2 -adrenoceptors - inhibits adenylate cyclase activity, resulting in reduced cyclic AMP production. Receptor-mediated inhibition of adenylate cyclase arises via the action of a pertussis toxin-sensitive, inhibitory G protein, G_i , although how adenylate cyclase is inhibited is not entirely clear. Inhibition may be mediated by the direct interaction of the $G_{i\alpha}$ subunit with adenylate cyclase and this may be

potentiated by the free $\beta\gamma$ subunits of G_i combining with any free $G_{s\alpha}$ subunit, thus terminating their stimulatory actions (Katada et al., 1986). It has also been proposed that the $\beta\gamma$ subunit may directly inhibit adenylate cyclase (Katada et al., 1986).

1.8.2 Mechanism of action of cyclic AMP

In mammalian cells, cyclic AMP exerts the majority of its biological actions through activation of a specific protein kinase, cyclic AMP-dependent protein kinase (protein kinase A). Protein kinase A exists in two major isoforms, types I and II, both are tetramers and are activated by cyclic AMP as follows:



where R represents the regulatory components and C represents the catalytic components (for review, Corbin et al., 1988). Binding of cyclic AMP results in the dissociation of the catalytic subunits, which then exert their actions through phosphorylation of cytosolic proteins. However, in some systems, for example, in olfactory cilia, cyclic AMP may exert a direct action on ion conductances, independently of protein kinase activation (Nakamura & Gold, 1987).

1.8.3 Degradation of cyclic AMP

Cyclic AMP degradation occurs via the action of phosphodiesterase enzymes which hydrolyse cyclic AMP to

5'-adenosine monophosphate (AMP). The main subtypes involved are type I (Calcium - calmodulin dependent), type II (cyclic GMP - stimulated), type III (cyclic GMP - inhibited) and type IV (cyclic AMP - specific), according to the classification of Beavo and Reifsnnyder (1990). In some tissues, for example, cardiac muscle, each of these subtypes is present, although this is not typical of all tissues and the distribution of the enzyme subtypes varies considerably.

1.8.4 Cyclic AMP and control of endothelial cell function

Endothelial cyclic AMP levels can be elevated by a number of agents including prostacyclin (Dembinska-Kiec et al., 1980; Hopkins & Gorman, 1981), β -adrenoceptor agonists (Buonassi & Venter, 1976; Hopkins & Gorman, 1981), forskolin (Adams Brotherton et al., 1982), adenosine (Legrand et al., 1990) and calcitonin gene-related peptide (CGRP; Crossman et al., 1987). Despite this, only a small number of studies have attempted to relate the changes in endothelial cyclic AMP levels to altered cell function. Initial studies suggested that elevation of cyclic AMP content induced by prostacyclin (PGI_2) served as a negative feedback mechanism to limit PGI_2 production (Hopkins & Gorman, 1981; Adams Brotherton & Hoak, 1982). However, later studies ascribed this inhibition to the cyclic AMP-independent, inhibitory actions of the phosphodiesterase inhibitor, 3-isobutyl-1-methyl xanthine, which was utilised during these studies (Adams Brotherton et al., 1982).

The possibility that cyclic AMP regulates endothelial production of EDRF has been investigated. Stimulation of EDRF production, following treatment with prostacyclin and forskolin, may result from elevation of endothelial cyclic AMP content (Shimokawa et al., 1988). Furthermore, two stimulants of adenylate cyclase, isoprenaline and CGRP, induce endothelium-dependent relaxation of rat aorta (Gray & Marshall, 1991 a, b) and this was inhibited by N^{G} -nitro-L-arginine, strongly suggesting the involvement of EDRF. Supporting these observations, vasodilation induced by β_2 -adrenoceptor agonists, in rat perfused hindquarters, was attenuated by an inhibitor of EDRF production, N^{G} -nitro-L-arginine methylester (L-NAME) (Gardiner et al., 1991). However, the inability of CGRP to enhance levels of cyclic GMP in co-culture systems of smooth muscle and endothelium, derived from bovine aorta, but to still increase cyclic AMP in both cell types, suggests that the actions of CGRP may not be endothelium-dependent in all cell types (Crossman et al., 1990).

1.9 Guanylate cyclase

1.9.1 Cyclic GMP

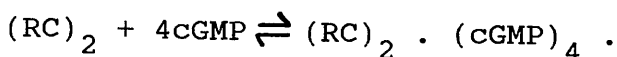
Cyclic GMP (guanosine - 3':5' cyclic monophosphate) formation is catalysed from guanosine triphosphate by two different enzymes, soluble guanylate cyclase and particulate guanylate cyclase (for review, Tremblay et al., 1988). Soluble guanylate cyclase is a cytosolic protein with a molecular weight of approximately 150kDa. Its

activation is promoted by a number of compounds including nitric oxide (Mittal & Murad, 1977), which may be EDRF (Palmer et al., 1987), and agents which act via the release of nitric oxide, such as the nitrovasodilators. Craven and De Rubertis (1978) first described the presence of a haem prosthetic group in guanylate cyclase; nitric oxide is thought to promote enzyme activation by binding to this haem moiety.

Particulate guanylate cyclase is distinct from soluble guanylate cyclase in that it is membrane associated and is typically activated by extracellular peptides, such as enterotoxins and atrial natriuretic factors (ANF) (Tremblay et al., 1988). How agents, such as ANF, activate particulate guanylate cyclase is not fully understood, although the ANF_A receptor and catalytic subunit appear to be tightly coupled (Chinkers et al., 1989) and may, in fact, be part of the same protein. A second, more abundant, class of ANF receptor, the ANF_C-receptor, exists although it is not coupled to guanylate cyclase (Leitman et al., 1986) and may represent a site for clearing ANF from the plasma.

1.9.2 Mechanism of action of cyclic GMP

Cyclic GMP exerts the majority of its actions through the activation of a protein kinase, termed cyclic GMP-dependent protein kinase. Activation of the enzyme may be represented by the following scheme:



Unlike cyclic AMP-dependent protein kinase, the regulatory subunits (R) and catalytic subunits (C) of cyclic GMP-dependent protein kinase do not dissociate upon binding of cyclic GMP (for review, Corbin et al., 1988). The phosphorylation of cellular substrates by this kinase mediates many of the actions of cyclic GMP, although the nucleotide may also act directly in some systems. For example, Fesenko and co-workers (1985) demonstrated the opening of sodium-permeable channels in the plasma membrane of retinal rods, following exposure to cyclic GMP.

1.9.3 Degradation of cyclic GMP

Degradation and inactivation of cyclic GMP occurs via the actions of phosphodiesterase enzymes which catalyse the hydrolysis of cyclic GMP to 5' guanosine monophosphate (GMP). The subtypes of phosphodiesterase which catalyse cyclic GMP hydrolysis are type I (calcium-calmodulin-stimulated) and type V (cyclic GMP-specific) (Beavo & Reifsnyder, 1990).

1.9.4 Cyclic GMP and control of endothelial cell function

Vascular endothelial cyclic GMP content can be elevated in response to both endogenously produced EDRF (Martin et al., 1988) and exogenously applied atrial natriuretic peptides (Leitman & Murad, 1986; Martin et al., 1988). However, whether or not cyclic GMP modulates endothelial cell

function is a subject of debate. The ability of 8 bromo cyclic GMP (Evans et al., 1988) and atrial natriuretic peptides (Hogan et al., 1989) to inhibit release of EDRF has lead to the proposal that elevation of endothelial cyclic GMP may serve as a negative feedback mechanism regulating EDRF production. However, this proposal is not supported by the recent work of Kuhn and co-workers (1991) who found that elevation of cyclic GMP content did not inhibit EDRF release from bovine aortic endothelial cells.

Doni and co-workers (1988) have proposed that nitric oxide may exert an inhibitory action on prostacyclin production in bovine aortic endothelial cells. This action is thought to be related to the ability of nitric oxide to elevate cyclic GMP, as the inhibitory effect was potentiated by zaprinast, a selective inhibitor of cyclic GMP phosphodiesterase (Doni et al., 1988). However, in previous studies, elevation of cyclic GMP, induced by atriopeptin II, 8 bromo cyclic GMP and sodium nitroprusside, appeared unable to modulate release of prostacyclin from either pig aortic endothelial cells (Martin et al., 1989) or human umbilical vein endothelial cells (Adams Brotherton, 1986).

In addition to possibly modulating release of EDRF and prostacyclin, elevation of cyclic GMP content, stimulated by atrial natriuretic peptides or nitrovasodilators, appears to inhibit both basal and thrombin-stimulated release of endothelin from human umbilical vein endothelial

cells (Saijonmaa et al., 1990). These findings are supported by the observations that N^{G} -nitro-L-arginine, an inhibitor of EDRF production, enhances endothelin production, while superoxide dismutase, which prolongs the half-life of EDRF, inhibits endothelin production from the endothelium of pig aorta (Boulanger and Lüscher, 1990). As endothelin is a potent vasoconstrictor, this action of cyclic GMP may represent an important mechanism by which EDRF, nitrovasodilators and atrial natriuretic peptides regulate vasoactive tone, in addition to their direct, inhibitory actions on blood vessels.

1.10 Interactive regulation of intracellular messenger systems and control of endothelial cell function

Little is known of the interactions which occur between second messenger pathways in the endothelial cell or how these interactions modify endothelial cell functions. Functional studies suggest that calcium acts as an important primary messenger in mediating certain rapid, cellular responses to extracellular stimuli. For example, the stimulated production both of EDRF and prostacyclin probably reflects the calcium-dependence of nitric oxide synthase (Meyer et al., 1989) and phospholipase A_2 (Van Den Bosch, 1980), respectively.

Evidence from other cell types indicates that other intracellular messengers, including protein kinase C, cyclic AMP and cyclic GMP, often exert their actions through the modulation of calcium mobilisation. For

example, calcium is the primary stimulus for contraction of smooth muscle (Bolzer, 1969) and for secretion and aggregation of platelets (Knight et al., 1982). Therefore, as agents which activate protein kinase C, or elevate cyclic nucleotide levels in these cells, have been found to inhibit calcium mobilisation (Meisheri & van Breemen, 1982; MacIntyre et al., 1985b; Collins et al., 1986; Itoh et al., 1988), this may represent the mechanism by which the second messengers affect cell function. Intracellular messengers may also augment calcium mobilisation. For example, cyclic AMP enhances calcium entry in several cell types, including hepatocytes (Poggioli et al., 1986), cardiac myocytes (Cachelin et al., 1983) and a pituitary cell line (Luini et al., 1985), while cyclic GMP also promotes calcium influx in pancreatic acinar cells (Pandol & Schoeffield-Payne, 1990). Hence, it is clear that cellular functions may be modulated by interactions between a number of second messengers, although the regulation of calcium mobilisation is likely to be the primary mechanism by which endothelial production of EDRF and prostacyclin is controlled. For example, tumour-promoting phorbol esters activate protein kinase C and have been shown to inhibit receptor-mediated production of EDRF, while having no effect on that induced by the calcium ionophore, A23187 (Weinheimer et al, 1986; Lewis & Henderson, 1987; Cherry & Gillis, 1988). This suggests that protein kinase C activation may inhibit EDRF release by inhibition of receptor-mediated mobilisation of calcium, the primary stimulus for EDRF production. Inhibition of EDRF and prostacyclin production by cyclic

GMP (Evans et al., 1988; Doni et al., 1988; Hogan et al., 1989) may also occur via the inhibition of calcium mobilisation. Therefore, by investigating how intracellular messengers, including protein kinase C, cyclic AMP and cyclic GMP, can control endothelial calcium mobilisation, we may gain a deeper insight into how these second messengers regulate endothelial cell function.

1.11 Scope of this investigation

In this study, endothelial $[Ca^{2+}]_i$ was measured using the fluorescent probe, fura-2. The contributions made by intracellular and extracellular calcium pools to agonist-induced elevations of $[Ca^{2+}]_i$ were assessed. Properties of the intracellular calcium stores, and how they are functionally linked to calcium in the extracellular fluid, were also investigated. Finally, the effects of protein kinase C stimulation and of elevation of cyclic AMP and cyclic GMP content were examined on agonist-induced mobilisation of intracellular and extracellular calcium.

CHAPTER 2

2.1 **Role of the endothelial barrier**

The endothelium and its underlying basement membrane constitutes an important barrier between the components of the blood and the tissues of the body. Although the endothelium allows the passage of small, non-polar molecules through the cell, it restricts the passage of charged molecules and high molecular weight solutes. Macromolecules must traverse the endothelial barrier by alternative pathways: for example, via inter-endothelial gaps, through the cells via transcellular pores or, in certain cases, by receptor-mediated endocytosis (Renkin, 1985). Alterations in the properties of this barrier are necessary for normal homeostasis but also underlie the development of pathological processes, such as inflammation and atherosclerosis.

Atherosclerosis is a pathological condition associated with smooth muscle proliferation, lipid disposition in the arterial wall and with the progressive narrowing of the vessel lumen. The processes underlying the development of this condition are complex and have been reviewed by Ross (1986) and by Munro and Cotran (1988). Endothelial dysfunction is believed to play a major role in the development of atherosclerosis and may occur as a result of injurious stimuli. These include elevated plasma levels of low density lipoprotein (LDL) or homocysteine, mechanical stress, cigarette smoke or, possibly, viral infection.

These stimuli are thought to reduce endothelial barrier function, facilitating entry of LDL into the blood vessel wall. LDL has been shown to promote monocyte adhesion to the endothelium, both in vivo and in vitro (Faggiato et al., 1984; Alderson et al., 1986). Release of the monocyte-derived cytokine, interleukin-1, may further compromise endothelial barrier function by both a direct action on the endothelium (Royall et al., 1989) and by enhancing monocyte adhesion to the endothelium, through stimulated expression of adhesion molecules (Bevilacqua et al., 1985). Endothelium dysfunction may also result in inhibition of EDRF and prostacyclin production, thus facilitating the adhesion of platelets and monocytes (Radomski et al., 1987c; Bath et al., 1991). Release of growth factors from endothelial cells, platelets and monocytes contributes to hypertrophy of smooth muscle in the blood vessel wall. When this occurs, at the sites of lipid deposition, the characteristic fibrous plaques of atherosclerosis are observed. Further development of these plaques, along with increased smooth muscle growth, leads to the narrowing of the blood vessel lumen which is associated with atherosclerosis.

Alterations in endothelial barrier function are of importance during the development of the inflammatory response, which may be defined as a coordinated response to local injury, consisting of blood vessel dilatation, invasion of tissues by leucocytes and the passage of blood proteins through post-capillary venule walls. Normally,

access of leucocytes and proteins to the tissue is restricted by the endothelial barrier, but permeability increases following the actions of a number of mediators, including histamine, kinins, prostaglandins and cytokines, leading to inflammatory oedema. In contrast to the slow alterations in endothelial barrier function which develop during atherosclerosis, those taking place during inflammation occur within minutes or hours and may, therefore, be more susceptible to modulation by rapid changes in the levels of endothelial intracellular messengers.

2.2 Development of inflammation

2.2.1 Role of haemodynamic changes in inflammation

Haemodynamic changes are perhaps the first observable occurrences during the development of inflammation and arise as a consequence of a marked increase in blood flow to the site of injury. This local increase in blood flow increases hydrostatic pressure, which forces fluid into the tissues and also allows for increased delivery of leucocytes to the site of injury. The importance of this increased blood flow in enhancing vascular permeability is illustrated by the ability of vasodilators, such as prostacyclin, to markedly enhance leakage induced by chemoattractants (Rampart & Williams, 1986). The rapid vasodilation induced during an inflammatory response may be modulated by the release of EDRF (Chander et al., 1988; Hughes et al., 1990) and may enhance oedema formation, as

inhibitors of EDRF production were found to inhibit substance P-induced oedema formation (Hughes et al., 1990). Calcitonin gene-related peptide and substance P, which are co-located in sensory nerves, may also be released during inflammation, resulting in vasodilation (Brain & Williams, 1989).

2.2.2 Role of the endothelial barrier in inflammation

The endothelium and its underlying basement membrane normally provides the major barrier to movement of blood components into the surrounding tissues. However, during the inflammatory response, changes occur in endothelial barrier function; these changes involve endothelial cell contraction, cytoskeletal re-organisation and, possibly, endothelial damage. The major site of the inflammatory response is the post-capillary venule (Majno et al., 1961; Svensjö et al., 1979). A number of inflammatory agents, such as serotonin, bradykinin and histamine, have been found to induce endothelial cell contraction (Majno et al., 1969), perhaps by a mechanism involving calcium, calmodulin and myosin light-chain kinase (Wysolmerski et al., 1990), although activation of protein kinase C may also be important (Antonov et al., 1986). The endothelial shape change and contraction results in rapid formation of inter-endothelial gaps (Majno et al., 1969) and the movement of fluid and plasma proteins into the extravascular space. Increased vascular permeability may also be promoted by cytokines, such as tumour necrosis factor, although these changes are much slower in onset

than those of the classical inflammatory mediators and occur over a number of hours (Stolpen et al., 1986). The endothelial barrier function may be further diminished by injury - via the dual actions of oxygen-derived free radicals and proteolytic enzymes released from neutrophils (Wedmore & Williams, 1981). Injured endothelial cells may retract or lyse, resulting in enhanced vascular leakage. This may result in enhanced blood stasis and, therefore, potentially greater leucocyte adhesion and migration.

2.2.3 Role of the endothelium in leucocyte adhesion and migration during inflammation

Endothelial-leucocyte adhesion is followed by diapedesis of the white blood cells across the endothelial barrier. Adhesion of neutrophils to endothelial cells occurs rapidly, in response to agents which raise endothelial cytosolic calcium, for example, thrombin and histamine, and may be associated with the translocation of the adhesion molecule, GMP-140, from the Weibel-Palade bodies to the plasma membrane (Hattori et al., 1989). Neutrophil adherence is facilitated by the endothelial production of platelet activating factor (PAF; Prescott et al., 1984). It is likely, that PAF facilitates neutrophil binding by inducing the up-regulation of neutrophil adhesion molecules, particularly members of the CD18 family (Tonnesen et al., 1989). Subsequent prolonged attachment of neutrophils to the endothelium may require interaction of these adhesion molecules with the endothelial adhesion molecules, ICAM-1 and ECAM-1 (Vevilaqua et al., 1987).

Expression of ECAM-1 appears to be optimal after 4 hours exposure to cytokines, such as interleukin-1 and TNF, and will bind both neutrophils and monocytes. These cytokines also promote the synthesis of interleukin-8, a low molecular weight cytokine, in a number of cell types. Interleukin-8 is chemotactic towards neutrophils and may also induce their degranulation (Matsushima & Oppenheim, 1989). It is likely that interleukin-8 is identical to the leucocyte adhesion inhibitor described by Gimbrone and co-workers (1989), which may switch neutrophil binding to a lower affinity adhesion molecule (for example, ICAM-1), thus aiding the diapedesis of the cells across the venule wall.

2.3 Endothelial barrier function - role of intracellular messenger systems

2.3.1 Calcium and inositol phosphates

Calcium mobilisation, resulting from the actions of inositol phosphates, may modulate both endothelial contraction and secretion and, therefore, regulate endothelial function during development of inflammation. A number of inflammatory mediators, including histamine, bradykinin and thrombin, inhibit endothelial barrier function in vivo (Majno & Palade, 1961; Marciniak et al., 1978; Svensjö et al., 1979) or in vitro (Killackey et al., 1986; Rotrosen & Gallin, 1986), probably via endothelial cell contraction (Majno et al., 1969; Laposata et al., 1983; Killackey et al., 1986).

Using an in vitro model in which human umbilical vein endothelial cells were grown on porous membranes, Rotrosen and Gallin (1986) demonstrated that histamine increases albumin transfer across endothelial monolayers with a similar concentration-dependence to the mobilisation of calcium. On the basis of this, they suggested that endothelial shape change or contraction may be dependent on changes in $[Ca^{2+}]_i$. This proposal is supported by the observations that calcium chelators inhibit histamine-induced vascular leakage (Liddell et al., 1981) in vitro and that A23187, a calcium ionophore, enhances albumin transfer across cultured endothelial monolayers (Shasby et al., 1985; Gudgeon & Martin, 1989). Thrombin- and platelet activating factor (PAF)-induced endothelial shape change (Laposata et al., 1983; Killackey et al., 1986; Garcia et al., 1986; Grigorian & Ryan, 1987) may also reflect the ability of these agents to increase endothelial $[Ca^{2+}]_i$ (Brock & Gimbrone, 1986; Jaffe et al., 1987).

In smooth muscle, calcium is the primary stimulus for induction of contraction (Bolzer, 1969), exerting its actions via the calcium-calmodulin activation of myosin light-chain kinase (MLCK). MLCK subsequently phosphorylates the 20kDa regulatory light chain subunit of myosin, resulting in smooth muscle contraction (Kamm & Stull, 1985). The presence of myosin and actin filaments has been demonstrated in the vascular endothelium (Becker & Nachman, 1973; Drenckhahn, 1983) and, therefore, a similar process may be involved in the development of endothelial

cell shape change and contraction. Furthermore, as endothelial levels of F-actin are reduced in response to calcium-mobilising agents (Rotrosen & Gallin, 1986), calcium may modulate endothelial cytoskeletal function by altering the activity of the F-actin fragmenting protein, gelsolin (Yin & Stossel, 1979).

Regulation of endothelial secretion by cytosolic calcium may also modulate induction of the inflammatory response. As described earlier, production of EDRF and prostacyclin is highly calcium-dependent (Singer & Peach, 1982; Long & Stone, 1985; Jaffe et al., 1987; Hallam et al., 1988a). Rapid elevation of $[Ca^{2+}]_i$ results in the rapid release of these mediators and a rapidly developing vasodilation during the initial stages of the inflammatory response.

Endothelial production of PAF is highly dependent on calcium influx, which probably exerts a regulatory action on the synthetic enzyme, phospholipase A_2 (Whatley et al., 1989). Hence, inflammatory agents may exert indirect actions on neutrophil activation and adhesion via the production of endothelium-derived PAF.

2.3.2 Protein kinase C

Protein kinase C activation results in the slow development of a long-lasting contraction in vascular smooth muscle, by a mechanism which is thought to involve enhanced calcium sensitivity of muscle contractile filaments (Itoh et al., 1988). Activation of protein kinase C may similarly induce

endothelial cell contraction and shape change, resulting in increased vascular leakage. In vitro studies have demonstrated that 4 β -phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C, induces shape change in human umbilical vein endothelial cells (Antonov et al., 1986) and bovine pulmonary artery endothelial cells (Grigorian & Ryan, 1987), and increases albumin transfer across monolayers of pig aortic endothelial cells (Gudgeon & Martin, 1989), although Olesen (1987) found no effect of PMA on vascular leakage from frog brain venules in vivo.

Activation of protein kinase C may further potentiate the inflammatory response by enhancing endothelial production of PAF (Whatley et al., 1989) thus facilitating neutrophil activation and adhesion to endothelial membranes.

2.3.3 Cyclic AMP

β -adrenoceptor agonists inhibit bradykinin - and histamine-induced vascular leakage from post-capillary venules (Svensjö et al., 1977, 1979; Marciniak et al., 1978). These inhibitory actions are likely to be due to a cyclic AMP-induced enhancement of barrier function. Evidence for this has been obtained in a number of in vitro studies which have demonstrated the ability of cyclic AMP elevating agents, such as isoprenaline and prostacyclin, to inhibit either endothelial albumin transfer or inter-endothelial gap formation (Laposata et al., 1983; Killackey et al., 1986; Mizuno-Yagya et al., 1987; Gudgeon & Martin, 1989). This action of the cyclic AMP-elevating

agents was associated with enhanced cell flattening and greater cell-to-cell contact (Bensch et al., 1983). In contrast to the above studies, adenylate cyclase activation has been shown to promote endothelial shape change and inter-endothelial gap formation in cultures of human umbilical vein endothelial cells (Antonov et al., 1986). However, as other studies have shown that cyclic AMP inhibits gap formation in these cells (Laposata et al., 1983; Killackey et al., 1986), the reasons for these differences are not apparent.

2.3.4 Cyclic GMP

Release of EDRF can enhance vascular leakage induced by inflammatory agents, such as substance P and histamine, although this action probably results indirectly from an increase in local blood flow (Chander et al., 1988; Hughes et al., 1990). It is not yet known whether changes in endothelial barrier function can be produced by EDRF by a direct effect on the endothelial cell during an inflammatory response. As described earlier, endothelial cyclic GMP content increases in response both to endogenously produced EDRF (Martin et al., 1988) and atrial natriuretic peptides (Leitman & Murad, 1986; Martin et al., 1988). By analogy with vascular smooth muscle where cyclic GMP and cyclic AMP induce relaxation (Itoh et al., 1985), it is possible that cyclic GMP, like cyclic AMP (Laposata et al., 1983; Bensch et al., 1983; Killackey et al., 1986), may induce endothelial cell relaxation and so enhance barrier function. However, it should be noted that

elevation of cyclic AMP, but not cyclic GMP, content enhances the integrity of gallbladder epithelial tight junctions (Duffey et al., 1981).

2.4 Role of intracellular messenger interactions in the regulation of endothelial barrier function

Progression of inflammatory oedema may be controlled by functional regulation at a number of different sites. For example, changes in blood flow will alter hydrostatic pressure and, therefore, alter vascular leakage. However, direct alterations in post-capillary endothelial barrier function may be one of the major mechanisms by which vascular leakage and, therefore, development of the inflammatory response may be modulated (Majno et al., 1961; Svensjö et al, 1979).

By comparison with other biological systems, alterations in endothelial barrier function are likely to be regulated by both synergistic and antagonistic interactions between intracellular messenger systems. However, it is not entirely clear where regulation occurs and how these interactions correlate with changes in endothelial barrier function. As described in section 2.3.1, elevations of $[Ca^{2+}]_i$ could be an important trigger for the development of endothelial contraction and the formation of inter-endothelial cell gaps. Agents which increase endothelial $[Ca^{2+}]_i$ may also increase endothelial hydrolysis of phosphoinositides (Derian & Moskowitz, 1986; Halldorsson et al, 1988), resulting in the concomitant

production of diacylglycerol. Diacylglycerol can directly activate protein kinase C and this enzyme may inhibit endothelial barrier function either alone or via the synergistic interaction with calcium which has been described in other cell systems (Nishizuka, 1986).

Several intracellular messenger pathways may be involved in the enhancement of endothelial barrier function and, as described in sections 2.3.3 and 2.3.4, cyclic AMP and cyclic GMP may have key roles in the regulation of this aspect of endothelial cell function. As both cyclic nucleotides inhibit smooth muscle contraction (Itoh et al., 1985), via a mechanism that involves inhibition of calcium mobilisation (Meisheri & van Breemen, 1982; Collins et al., 1986), it is important to determine whether they may exert similar actions on calcium mobilisation in the endothelium and whether these actions relate to their functional effects in the endothelium. Alternatively, cyclic AMP and cyclic GMP may exert their functional actions via a different mechanism - for example, via inhibition of the actions of protein kinase C (Rasmussen et al., 1984).

2.5 Scope of this investigation

In this study, an in vitro model of endothelial barrier function was established, using bovine pulmonary artery endothelial cells, and the effects of activating several intracellular messenger pathways examined. Specifically, the second messenger pathways considered were calcium, protein kinase C, cyclic AMP and cyclic GMP. The

mechanisms underlying the alterations in endothelial barrier function were examined. Specifically, the effects of the cyclic nucleotides, cyclic AMP and cyclic GMP, and of stimulation of protein kinase C on agonist-induced calcium mobilisation were examined.

METHODS

CHAPTER 3

3.1 Isolation of Primary Cultures of Endothelial Cells

3.1.1 Isolation of bovine aortic endothelial cells (BAEC)

At a local abattoir, bovine thoracic aortae were removed shortly after death (approximately 5 minutes) and flushed with sterile saline containing benzyl penicillin (100U ml^{-1}) and streptomycin ($100\mu\text{g ml}^{-1}$). The end of each vessel, originally proximal to the aortic arch, was ligated and the distal end subsequently cannulated with a 60ml syringe and adaptor containing the same sterile saline. This saline was then infused into the lumen of the aorta and the vessel transported to the laboratory.

At the laboratory, all subsequent work was undertaken in a laminar flow hood (Flow laboratories). The aortic intercostal arteries were cleared of adhering fat and ligated with surgical thread. 20ml of a sterile collagenase solution (Type II, Sigma; 0.1% in Dulbecco's modification of Eagle's medium (DMEM)) was then infused into the lumen. The vessel was then incubated at 37°C in an atmosphere of 5% CO_2 /95% air for 25 min. At the end of this incubation period, the aorta was gently massaged and the resulting cell suspension collected. To collect any remaining cells, sterile saline (20ml) was infused into the lumen and then removed. The resulting cell suspensions were centrifuged (200g; 4 min; 10°C). The supernatants were decanted and the cell pellets resuspended in DMEM

containing foetal calf serum (10%), newborn calf serum (10%), glutamine (4mM), benzyl penicillin (200U ml^{-1}) and streptomycin ($200\mu\text{g ml}^{-1}$) (referred to as complete medium throughout the rest of the text). These suspensions of BAEC from each aorta were combined and centrifuged again (200g ; 4 min; 10°C). The resulting cell pellet was resuspended in 50ml of complete medium and seeded into 2 or 3 80cm^2 culture flasks (Gibco). The flasks were then placed in an incubator (Flow laboratories) at 37°C in an atmosphere of 5% CO_2 and 95% air. The culture medium was changed the day following the initial isolation and every 2-3 days subsequently. Figures 3.1.a and 3.1.b show light micrographs of BAEC one day after isolation and at confluence (3 days), respectively. The cells demonstrated the characteristic endothelial "cobblestone" morphology.

3.1.2 Isolation of bovine pulmonary artery endothelial cells (BPAEC)

At a local abattoir, bovine pulmonary arteries were removed and flushed with sterile saline containing benzyl penicillin (100U ml^{-1}) and streptomycin ($100\mu\text{g ml}^{-1}$). The artery was then transferred to a plastic container which had been sterilised using 100% ethanol the previous day and which contained the above sterile saline. The container was then transferred to the laboratory and subsequent procedures undertaken in a laminar flow hood (Flow laboratories). The end of the artery, which was originally proximal to the heart, was ligated and, at the other end, one of the two branches of the artery was clamped with 2

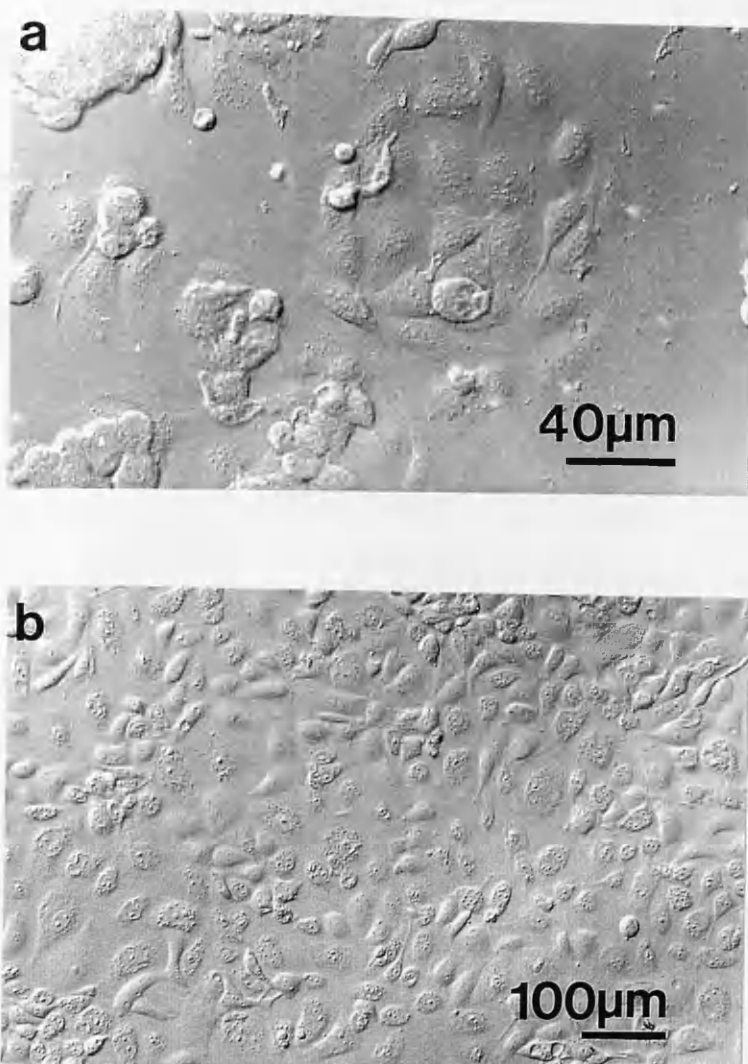


Figure 3.1 Differential interference contrast micrographs of primary cultures of bovine aortic endothelial cells, isolated by collagenase treatment. The cells are isolated as clumps, and adhere to the underlying substrate within 24 hours (a). They subsequently divide and multiply to form a strict monolayer which displays the characteristic cobblestone appearance of the vascular endothelium. A confluent monolayer obtained 3 days after isolation is shown (b).

artery forceps. The second branch was then cannulated with a syringe and adaptor containing 10ml of sterile collagenase solution (type II, Sigma; 0.1% in DMEM). The collagenase solution was infused into the lumen and the vessel incubated at 37°C for 25 minutes in an atmosphere of 5% CO₂/95% air. At the end of this incubation period the artery was gently massaged and the resulting cell suspension collected. Sterile saline (10ml) was then infused into the vessel lumen and the remaining cells harvested. Following centrifugation (200g; 4 min; 10°C), the resulting cell pellet was resuspended in 50ml of complete medium and the cell suspension was seeded into 2 or 3 80cm² culture flasks (Gibco). With BPAEC, thymidine (10⁻⁵M) was included in the complete medium, as this supplement was found to aid healthy cellular growth. Use of this supplement has also been proposed by Laskey and co-workers (1990). The flasks were then placed in an incubator (Flow laboratories) in an atmosphere of 5% CO₂ and 95% air. The medium was changed the day after isolation and every 2-3 days subsequently. Confluence was reached after 3-6 days. The cells displayed the characteristic endothelial "cobblestone" morphology.

3.2 Measurement of Calcium Using Fura-2

3.2.1 Principles of measuring calcium concentrations ([Ca²⁺]) using quin 2 and fura-2

Development of the fluorescent calcium indicator, quin 2 (Figure 3.2), by Tsien and co-workers (1982a, b) allowed

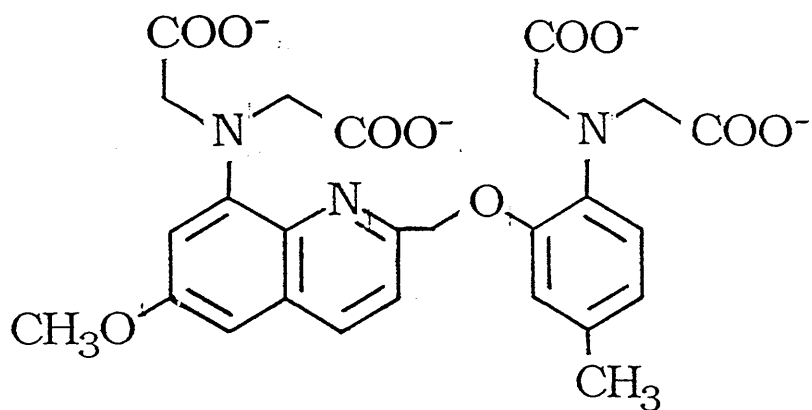


Figure 3.2 Structure of quin 2 acid.

the first measurement of cytoplasmic calcium concentration ($[Ca^{2+}]_i$) in populations of small mammalian cells. Quin-2 has calcium-binding properties, as its structure is derived from EGTA, a calcium chelator, and fluorescence properties, due to attached fluorophores. The suitability of quin 2 to act as a calcium indicator stems from its ability to sense changes in $[Ca^{2+}]_i$ in the physiological range (nM- μ M). Although quin 2 itself cannot enter cells, it may be loaded into cells using a membrane-permeant ester derivative. This is hydrolyzed by cytosolic esterases to the membrane-impermeant quin 2 anion, thus leaving the fluorescent dye trapped in the cytosol. $[Ca^{2+}]_i$ may be evaluated by excitation at a wavelength of 339nm with fluorescence emission collected at 492nm, with increases in fluorescence being proportional to increases in $[Ca^{2+}]_i$.

Although the development of quin 2 was a major step forward, its use has several limitations. For example, quin 2 does not undergo much of a shift in excitation (Figure 3.3) or emission wavelength upon the binding of calcium. Hence, as measurement of $[Ca^{2+}]_i$ with quin 2 is wholly dependent on absolute fluorescence changes, errors may arise due to changes in fluorescence which are independent of changes in $[Ca^{2+}]_i$. These variable factors include cellular concentrations of quin 2, cell thickness in the beam pathway, photobleaching and the intensity and stability of the illumination source. Furthermore, the extinction coefficient and quantum yield of quin 2 are low and this necessitates a high cytosolic dye concentration,

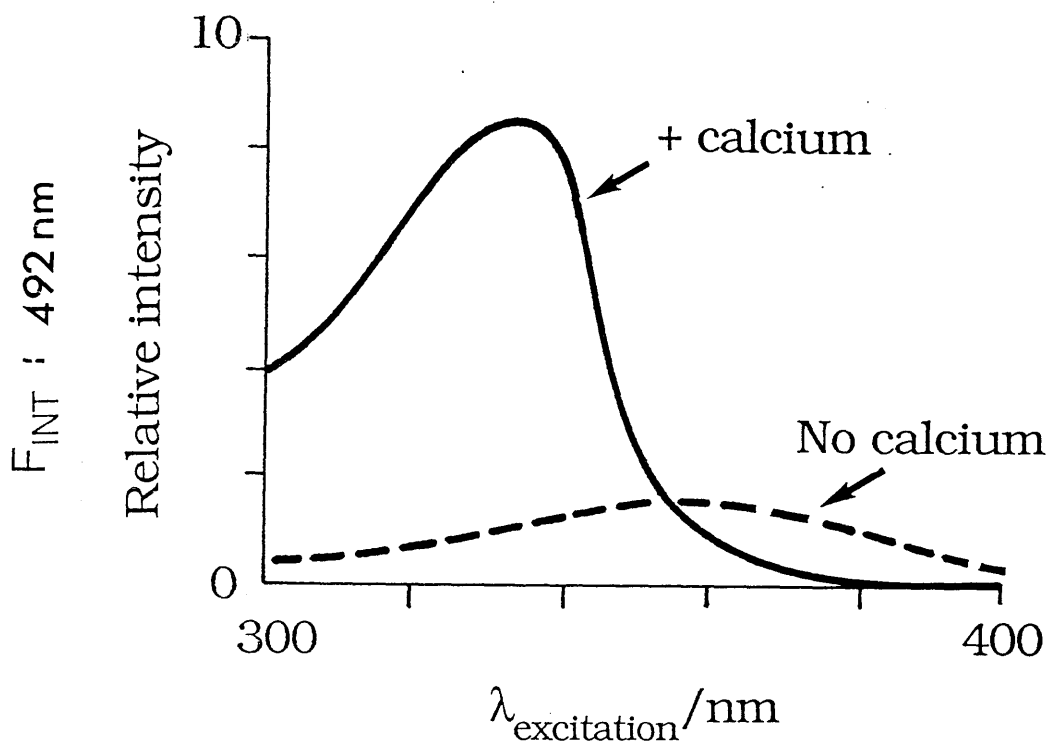


Figure 3.3 Excitation spectrum illustrating the spectral characteristics of quin 2 acid in the presence of 1mM calcium (solid line) and in the absence of calcium (broken line). Emission was collected at 492nm.

which may result in the buffering of calcium transients. The high affinity of quin 2 for calcium allows accurate measurement of $[Ca^{2+}]_i$ near resting levels of 100nM but, at near micromolar levels, the dye approaches saturation and resolution of the signal is lost.

More recently, the development of a new class of calcium indicators, including fura-2, has overcome some of the problems associated with quin 2 (Grynkiewicz et al., 1985). In this study, measurement of calcium was routinely undertaken using the fluorescent probe, fura-2. Fura-2, like quin 2, has the ion binding properties of bivalent ion chelators (e.g. EGTA) in addition to attached fluorophores. The structure of fura -2 is illustrated in Figure 3.4. The improved ability of fura-2 over quin 2 depends upon its ability to alter its spectral properties and to increase in fluorescence emission upon calcium binding. Like quin 2, fura-2 is a charged molecule at physiological pH and does not readily pass through the cell membrane. However, the incorporation of five acetoxymethylester (AM) groups onto the carboxylate functional groups of fura-2 produces a molecule which will readily diffuse across the cell membrane; the structure of fura-2/AM is illustrated in Figure 3.5. The hydrolysis of the AM functional groups leads to the cytosolic production of calcium-sensitive fura-2. This process is illustrated schematically in Figure 3.6. In the absence of calcium, fura-2 fluorescence emission (measured at 509nm) consists of a large, featureless band with peak fluorescence produced at an

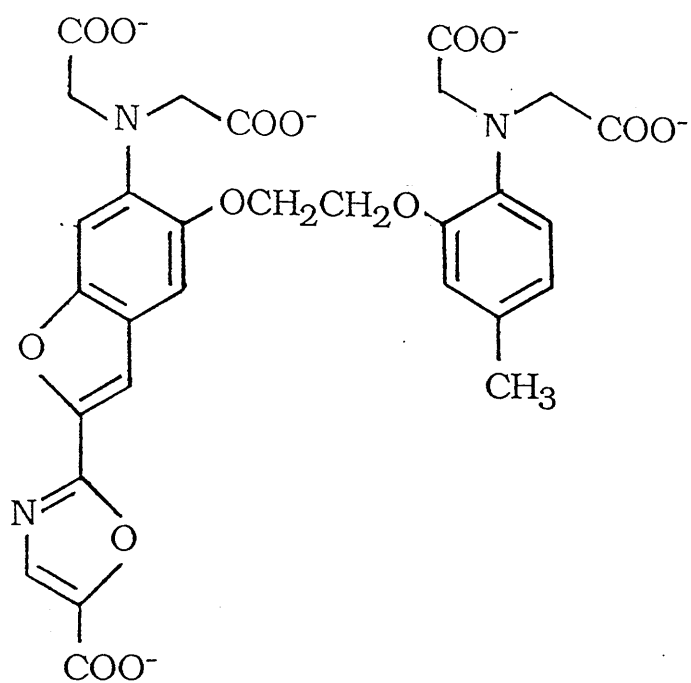


Figure 3.4 Structure of fura-2 acid.

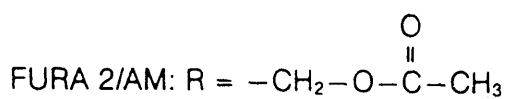
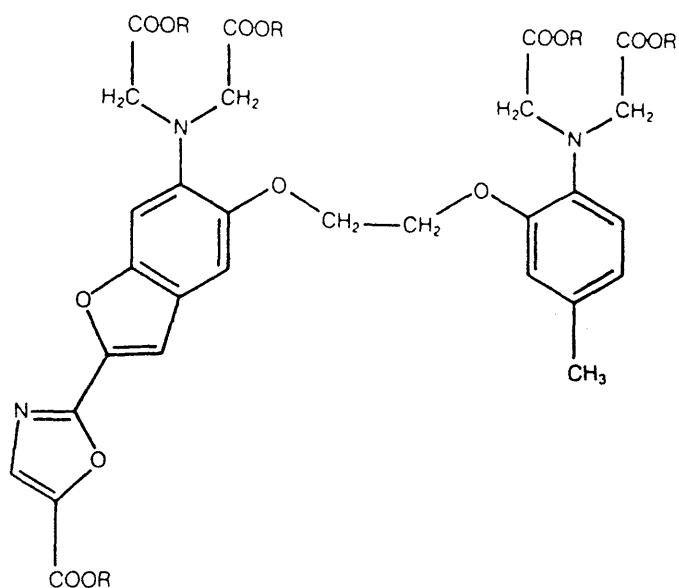


Figure 3.5 Structure of fura-2 penta-acetoxymethylester (fura-2/AM)

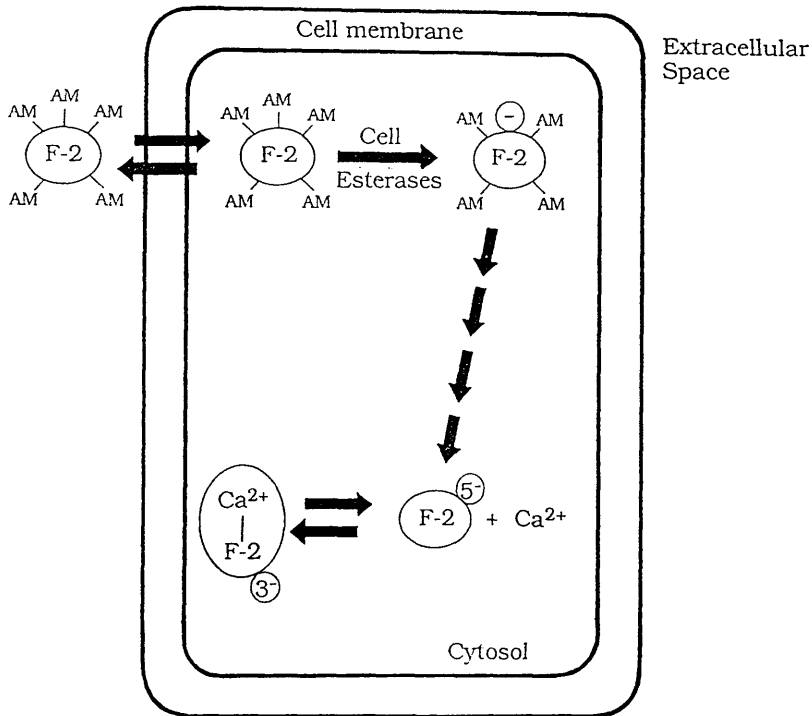


Figure 3.6 Diagrammatic representation of the cellular loading of fura-2. The membrane-permeant fura-2 pentaacetoxymethylester (fura-2/AM) enters the cytoplasm and is hydrolysed by cytosolic esterases to yield the calcium-sensitive form of the indicator, fura-2 acid, which is trapped within the cells.

excitation wavelength of 360-370nm (Figure 3.7). Binding of calcium shifts the peak wavelength of the fura-2 excitation spectrum to 340-350nm (Figure 3.7). Saturation of the dye with calcium results in a 3-fold rise in fluorescence emission excitation at 340nm and a 10-fold fall in fluorescence emission following excitation at 380nm. The calcium concentration is defined absolutely by the ratio of emissions obtained following excitation at 340nm (F_{340}) and 380nm (F_{380}). These changes provide the basis for the measurement of calcium using ratio mode (Grynkiewicz et al., 1985). Using this procedure, as the calcium concentration ($[Ca^{2+}]$) increases, the ratio (R) of the dye's fluorescence intensities, F_{340} and F_{380} , also increases. The experimental ratio (R) is independent of potentially variable factors such as the degree of dye loading or instrumental factors, changes in cell thickness and photobleaching.

Experimentally derived ratios (R) may then be inserted into the equation derived by Grynkiewicz et al. (1985):

$$\text{Equation 1: } [Ca^{2+}] = K_d \cdot \frac{(R - R_{\min})}{(R_{\max} - R)} \cdot \frac{(Sf2)}{(Sb2)}$$

Where K_d is the dissociation constant for the Ca^{2+} - fura-2 complex; R_{\max} is the maximal ratio ($F_{340} : F_{380}$) obtained in saturating levels of calcium; R_{\min} is the minimal ratio ($F_{340} : F_{380}$) obtained in zero calcium, Sf2 is the fluorescence emission measured at an excitation wavelength

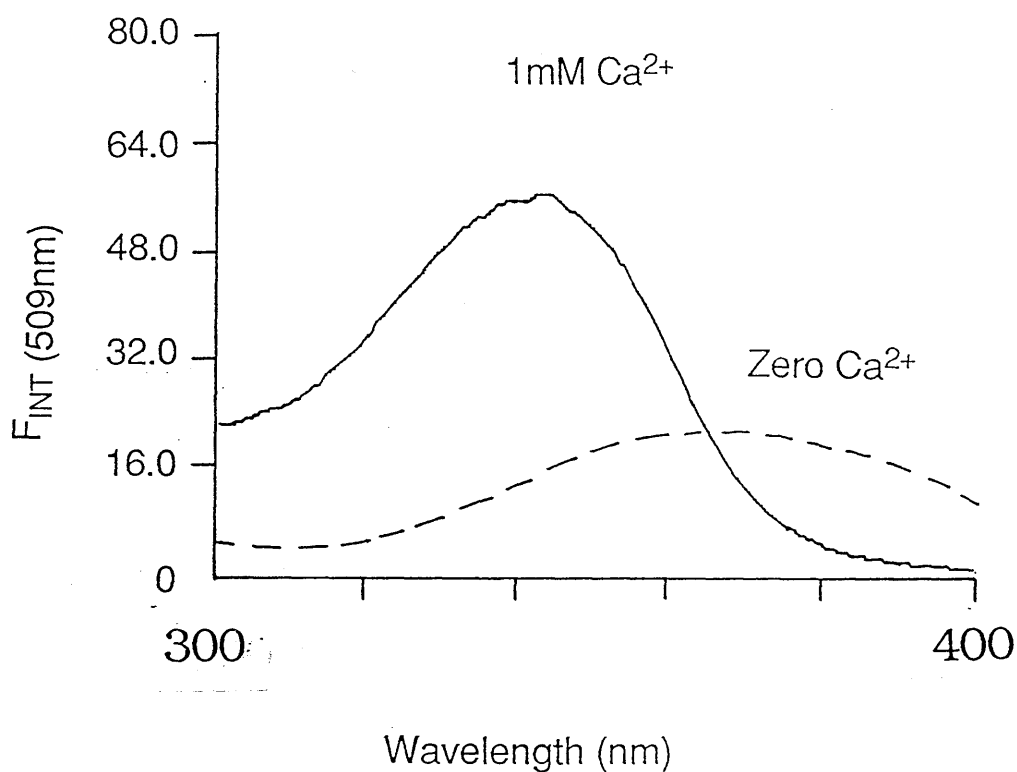


Figure 3.7 Excitation spectrum of fura-2 acid in the presence of saturating levels of calcium. HEPES (10mM)-buffered Krebs containing $0.25\mu\text{M}$ fura-2 acid and 1mM calcium was placed in a quartz cuvette. This level of calcium saturated the fura-2 calcium binding sites and the resultant excitation spectrum is shown by the solid line. Fluorescence emission was collected at 509nm. Subsequent addition of EGTA (40mM) chelated calcium and converted fura-2 to the calcium-free form. The resultant excitation spectrum is shown by the broken line. Fluorescence emission was again collected at 509nm.

of 380nm in the absence of calcium and Sb_2 is the fluorescence emission measured at an excitation wavelength of 380nm in the presence of saturating calcium. When these calibration values are required for either fura-2 in solution or for fura-2-loaded cells, it is desirable to experimentally calculate these parameters for a given fluorimeter, due to possible instrumental variability.

To calculate these parameters, Krebs containing (mM) : NaCl 118, KCL 4.8, $MgSO_4$ 1, $NaHCO_3$ 2.4, glucose 11, HEPES 10, $CaCl_2$ 1 and fura-2 0.00025 was added to a quartz cuvette and placed in a Perkin-Elmer LS-3B fluorimeter. The cuvette was thermostatically controlled at 37°C and the solution was stirred continuously. Data was collected and processed via an IBM-PCAT and fluorescence emission was measured at 509nm at all times. A diagrammatic representation of the experimental set-up is illustrated in Figure 3.8. At this calcium concentration (1mM), the fura-2 dye was saturated with calcium and R_{max} (the $F_{340} : F_{380}$, in saturating calcium) was obtained. Figure 3.7 illustrates the excitation spectrum of fura-2 obtained experimentally, in buffer containing 1mM calcium. Addition of EGTA (40mM) chelates calcium and therefore removes calcium from the fura-2 binding sites. The excitation spectrum in calcium-free conditions is illustrated in Figure 3.7, and from this, R_{min} ($F_{340} : F_{380}$ in zero Ca^{2+}) was obtained. These derived values for R_{max} and R_{min} require to be corrected as they include a contribution from auto-fluorescence, a background fluorescence derived from

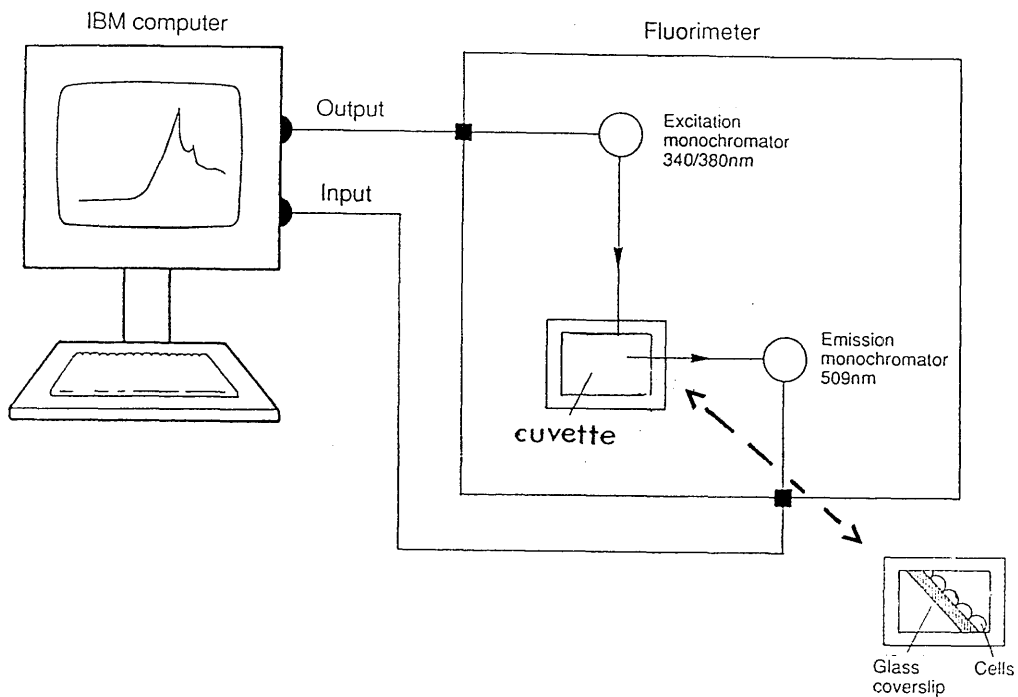


Figure 3.8 Diagrammatic representation of the experimental apparatus for measuring $[Ca]$ in solution or $[Ca^{2+}]_i$ in the endothelium. The cuvettes are interchangeable as denoted by the double-headed arrow. When endothelial $[Ca^{2+}]_i$ was measured, fura-2-loaded monolayers of endothelial cells were suspended in a quartz cuvette containing HEPES (10mM)-buffered Krebs and maintained at $37^\circ C$ in a Perkin-Elmer LS-3B fluorimeter. The excitation monochromator was under computer control (IBM-PCAT) and was driven between 340nm and 380nm every 3.8s. Fluorescence emission data was collected at 509nm and stored in the computer. At the end of the experiment, $[Ca^{2+}]_i$ was calculated from the fluorescence data.

the cuvette, buffer solutions and fluorimeter optics. Cells, if included, will also contribute to auto-fluorescence. To obtain correct values for R_{\max} and R_{\min} , it is important to calculate this auto-fluorescence. This can be done utilising the ability of manganese (Mn^{2+}) to quench fura-2 fluorescence (Grynkiewicz et al., 1985). Therefore, at the end of each calibration, Mn^{2+} (200mM) is added to the fura-2/EGTA containing buffer to quench fura-2 fluorescence. AF_{340} and AF_{380} , the resultant auto-fluorescence values at 340nm and 380nm, respectively, were then subtracted from the raw F_{340} and F_{380} data and the corrected ratios for R_{\max} and R_{\min} subsequently calculated. Using this procedure, the following experimentally-derived values were obtained. R_{\max} was 15.3 ± 0.1 (n=4) and R_{\min} was 0.73 ± 0.01 (n=4). Sf2/Sb2 is the ratio of the corrected F_{380} in zero calcium to the corrected F_{380} in saturating calcium and was 5.65 ± 0.06 (n=4).

3.2.2 Calculation of $[Ca^{2+}]$ in experimental buffers

Certain experimental protocols required the use of buffers containing low concentrations of calcium and it was important to determine precisely the free calcium concentrations ($[Ca^{2+}]$) present. The calcium contents of these buffers were (a) nominally calcium-free, (b) nominally calcium-free, plus 0.5mM EGTA, (c) 1mM calcium plus 2mM EGTA and (d) 1mM calcium plus 5mM EGTA. Fura-2 is able to detect changes in $[Ca^{2+}]$ in the range 10nM - 3 μ M (Grynkiewicz et al., 1985) and was therefore used to

calculate $[Ca^{2+}]$ in these situations. In each case, the Krebs buffer contained the following (mM) : NaCl 118, KCL 4.8, $NaHCO_3$ 2.4, $MgSO_4$ 1, glucose 11 and HEPES 10. Each Krebs solution was placed in a quartz cuvette, maintained at 37°C, and stirred continuously. Fura-2 acid (0.25 μ M) was then introduced into the cuvette and uncorrected F_{340} and F_{380} values were obtained. This fluorescence data was stored in the computer (IBM-PCAT). Subsequent addition of manganese chloride (40mM) quenched the fura-2 fluorescence leaving the auto-fluorescence. These values (AF_{340} , AF_{380}) were then subtracted from the F_{340} and F_{380} values obtained for the experimental ratio, to provide a corrected ratio (R). The values for R_{max} , R_{min} and Sf2/Sb2 (as determined in section 3.2.1) were substituted into equation 1 (K_d was assumed to be 224nM at 37°C [Grynkiewicz et al., 1985]). An accurate estimate of the actual calcium concentration present in each of the Krebs buffers could then be calculated, using the corrected $F_{340}:F_{380}$ ratios (R).

$[Ca^{2+}]$ <u>Added</u>	<u>[EGTA]</u> <u>Added</u>	Actual $[Ca^{2+}]$ <u>present</u>
Nominally " Ca^{2+} -free"	-	659 \pm 3nM (n=4)
Nominally " Ca^{2+} -free"	0.5mM	12 \pm 1nM (n=4)
1mM Ca^{2+}	2mM	506 \pm 30nM (n=4)
1mM Ca^{2+}	5mM	101 \pm 2nM (n=4)

3.2.3 Preparation of endothelial cells for fura-2 loading

Upon attaining confluence (3-5 days after initial isolation, primary cultures of BAEC or BPAEC were passaged, i.e. the culture medium was decanted and the flask (80cm²) of endothelial cells was rinsed twice with 20ml of sterile

saline. 10ml of an isotonic solution of trypsin (0.05%) and EDTA (0.02%) (Flow laboratories) was introduced into the flask and the cells incubated at 37°C until all of the cells had detached (usually 2-4 min). The cell suspension was combined with 2ml of newborn calf serum (NBCS, Gibco) to neutralise the action of the trypsin and the suspension washed by spinning twice at 200g for 4 min (10°C) followed by suspension in whole medium. Cells were finally resuspended in 10ml of complete medium. As stated previously (section 3.1.2), thymidine (10^{-5}M) was included in the culture medium for BPAEC. Volumes of 0.5ml of the cell suspension were seeded onto sterile glass coverslips (11x42mm; No.2) which were placed in individual petri dishes (60x15mm; Gibco). After 60 min, the cells had attached to the coverslip and 5ml of complete medium was added to each petri dish. This medium was replaced every 3 days. Figures 3.9a and 3.9b illustrate the cell density of BAEC 1 day and 3 days after seeding, respectively. The cells were used upon reaching confluence, typically after 3-4 days.

3.2.4 Loading of endothelial cells with fura-2 and measurement of $[\text{Ca}^{2+}]_i$

Confluent endothelial monolayers, grown on coverslips as indicated above 3.2.3 were placed in a petri dish containing 4ml of HEPES(20mM)-buffered DMEM (Northumbria Biologicals, Cramlington, UK) containing 1% bovine serum albumin (BSA; Fraction V, Sigma) and $2\mu\text{M}$ fura-2/AM and incubated at 37°C for 45 min. Placing the petri dish on an

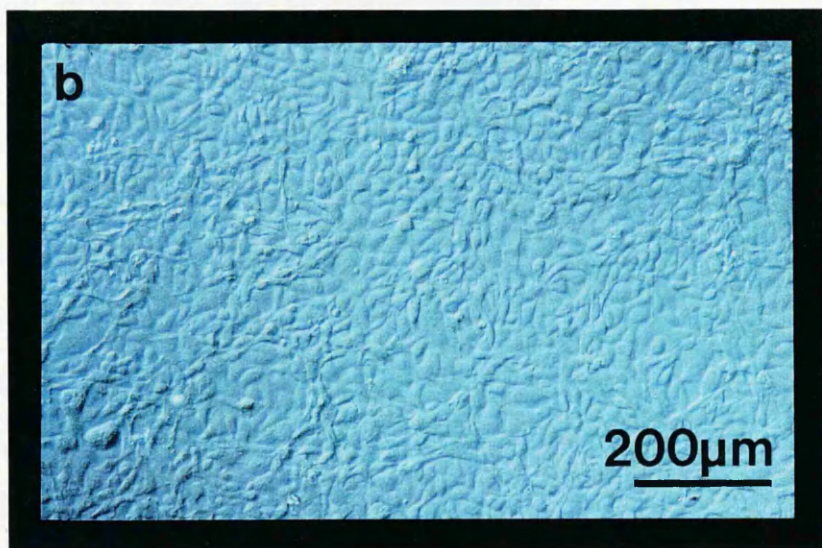
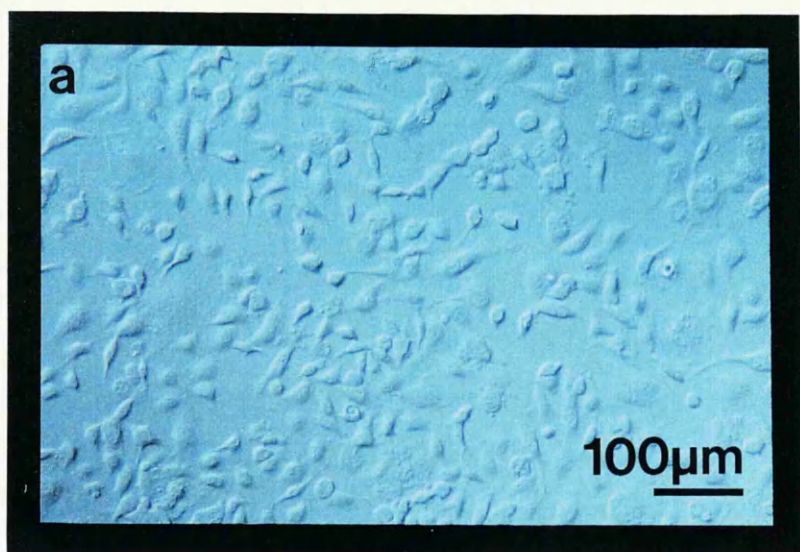


Figure 3.9 Differential interference contrast micrographs of first passage bovine aortic endothelial cells, cultured on the glass coverslips used for fluorescence studies. The micrographs illustrate (a) cells at low density, 24 hours after passaging and (b) a confluent monolayer of cells, 3 days after passaging.

orbital shaker (Luckham), at a low speed setting, ensured that the medium was mixed continuously. At the end of this loading period, the cells were transferred to HEPES-buffered Krebs containing (mM) :NaCl 118, KCL 4.8, NaHCO₃ 2.4, MgSO₄ 1, HEPES 10 and glucose 11. CaCl₂ was added at the concentrations indicated in the Results. In experiments where nickel chloride (NiCl₂) was used, NaHCO₃ was omitted and MgSO₄ replaced with MgCl₂ (1mM) to avoid the precipitation of these salts with nickel. The cells remained in the HEPES(10mM)-buffered Krebs for 20 min at room temperature; this allowed extracellular fura-2/AM to be washed from the cells and for intracellular fura-2/AM to be hydrolyzed to the calcium-sensitive fura-2 acid. Fura-2-loaded endothelial monolayers were suspended across the diagonal of a quartz cuvette containing 4ml of the same HEPES(10mM)-buffered Krebs. The cuvette was transferred to a Perkin-Elmer LS-3B fluorimeter and the coverslip positioned at an angle of 30° to the excitation beam. This angle minimises the scattering of incident light towards the emission monochromator. The cuvette was maintained at 37°C and stirred continuously throughout the experiment. The excitation monochromator was computer-driven (IBM-PCAT) and alternated between 340nm and 380nm every 3.8s. Fluorescence emission was collected at 509nm. Drugs were added to the cuvette in small volumes (typically 4-8μl). Data collection was stopped immediately before and directly after addition of drugs, so as to reduce artefacts associated with exposing the emission monochromator to ambient light. At the end of each experimental run,

background auto-fluorescence was obtained by the method of Hallam et al. (1988a), which requires the addition of ionomycin (1 μ M) to permeabilise the cells to divalent cations and the subsequent addition of MnCl₂ (2mM) to quench intracellular fura-2 fluorescence. The corrected fura-2 fluorescence values obtained at 340nm were divided by those obtained at 380nm, giving a corrected ratio (R), as indicated in section 3.2.1.

3.2.5 Calibration of endothelial [Ca²⁺]_i

Having obtained corrected values for the ratio (R), [Ca²⁺]_i was then calculated by the computer using the equation of Grynkiewicz et al. (1985):

$$\text{Equation 1: } [\text{Ca}^{2+}]_i = K_d \cdot \frac{(R - R_{\min})}{(R_{\max} - R)} \cdot \frac{(\text{Sf2})}{(\text{Sb2})}$$

It was important to experimentally obtain values for R_{max}, R_{min}, Sf2 and Sb2 in fura-2-loaded endothelial cells, as the spectral characteristics of the dye in the cytoplasm may differ from those obtained in a simple buffer solution.

The fluorescence properties of fura-2 were determined in endothelial cell suspension rather than in cells on coverslips as the low calcium concentrations required to obtain R_{min} would have resulted in detachment of cells. Cell suspensions were obtained by treating a flask of confluent BAEC with 10ml of an isotonic solution of trypsin (0.05%) and EDTA (0.02%) (Flow laboratories, Irvine, UK).

Following detachment of the endothelial cells, typically after 2-4 minutes, the effects of trypsin were neutralised by the addition of 2ml of NBCS. After twice spinning (200g; 4 min; 10°C) the cells and resuspending in whole medium, one quarter of the cell suspension (approximately 3×10^6 cells) was spun again and resuspended in 1ml of HEPES(20mM)-DMEM containing 1% BSA and $2\mu\text{M}$ fura-2/AM. After incubation at 37°C for 45 min, the cell suspension was diluted ten-fold with HEPES(20mM)-DMEM and, after spinning (200g; 4 min; 10°C), the cells were resuspended in 10ml of Krebs containing (mM) : NaCl 118, KCl 4.8, MgSO_4 1, NaHCO_3 2.4, glucose 11, HEPES 10 and CaCl_2 1.8. Following incubation of the fura-2-loaded cell suspension for 20 min at room temperature, the cells were spun again (200g; 4 min; 10°C) to remove any extracellular fura-2, resuspended in 2ml of the same HEPES(10mM)-buffered Krebs and transferred to a quartz cuvette. The cuvette was maintained at 37°C in the fluorimeter and stirred continuously. The trace from a typical experimental calibration run is shown in Figure 3.10. The excitation wavelength was alternated between 340nm and 380nm every 3.8 seconds and fluorescence emission collected at 509nm. Addition of ATP($30\mu\text{M}$), a receptor-mediated agonist, increased fluorescence following excitation at 340nm (F_{340}) and reduced fluorescence following excitation at 380nm (F_{380}). Ionomycin ($8\mu\text{M}$), a bivalent cation ionophore which translocates calcium across the cell membrane leading to saturation of the fura-2 with calcium, increased F_{340} to a maximum value and reduced F_{380} to a minimal value. After

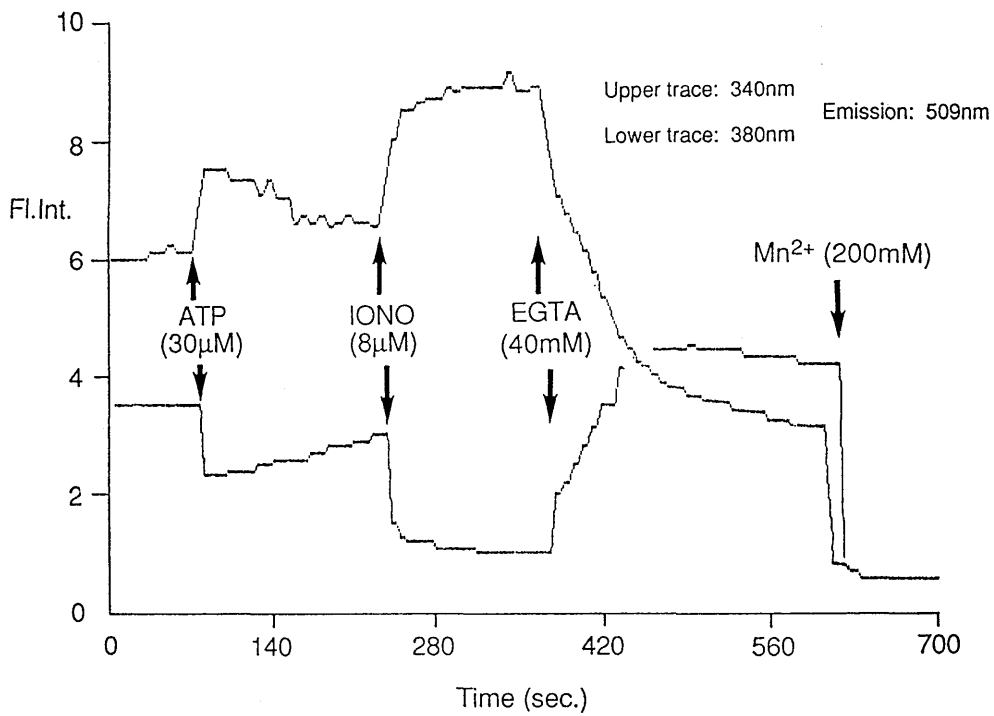


Figure 3.10 Experimental trace illustrating the fluorescence changes which occur during an experimental calibration. Approximately 3×10^6 BAEC were loaded with fura-2, suspended in 2ml of HEPES (10mM)-buffered Krebs in a quartz cuvette and maintained at 37°C in a Perkin-Elmer LS-3B fluorimeter. The excitation wavelength was alternated between 340nm and 380nm every 3.8s and fluorescence emission was collected at 509nm. Addition of ATP(30μM) increased fluorescence following excitation at 340nm(F_{340}) and reduced fluorescence following excitation at 380nm (F_{380}). Ionomycin (8μM), a divalent cation ionophore, increased F_{340} to a maximum level and reduced F_{380} to a minimum level. At this point, the ratio $F_{340}:F_{380}$ yielded the R_{\max} for fura-2. Addition of EGTA (40mM) stripped calcium from fura-2 binding sites, resulting in F_{340} falling to a minimum level and F_{380} rising to a maximum level. At this point, the ratio $F_{340}:F_{380}$ yielded the R_{\min} for fura-2. Addition of manganese chloride (Mn^{2+} ; 200mM) quenched fura-2 fluorescence leaving only background auto-fluorescence. This could then be subtracted from the F_{340} and F_{380} values to obtain a corrected ratio (R) for each experimental time-point.

the subtraction of auto-fluorescence, the ratio $F_{340}:F_{380}$, at this time point, yields R_{\max} . Addition of EGTA (40mM) chelated extracellular calcium and stripped intracellular calcium from fura-2 binding sites. F_{340} fell to a minimal value while F_{380} rose to a maximum value. After subtraction of auto-fluorescence, the ratio $F_{340}:F_{380}$ at this point yielded R_{\min} . The EGTA stock solution also contained sodium hydroxide (2M) to ensure that the final pH of the HEPES(10mM)-buffered Krebs was greater than 8.3. This high pH aids the dissociation of calcium ions from intracellular fura-2 binding sites. $MnCl_2$ (200mM) was then added to quench fura-2 fluorescence and yield auto-fluorescence values at 340nm and 380nm; these were then subtracted from the raw fluorescence data to yield corrected R_{\max} , R_{\min} , Sf2 and Sb2 values. The following values were obtained: R_{\max} 16.3 ± 1.2 (n=4), R_{\min} 0.81 ± 0.05 (n=4) and Sf2/Sb2 7.3 ± 0.6 (n=4). These calibration parameters are similar to those obtained by Hallam and Pearson (1986) and were used in all subsequent experiments.

3.3 Endothelial Barrier Function

3.3.1 Evaluation of endothelial barrier function

The vascular endothelium acts as a barrier to the passage of high molecular weight molecules. Barrier function was investigated using an in vitro model, represented diagrammatically in Figure 3.11, in which endothelial cells were grown on Costar Transwells. This is a two chamber model, consisting of an upper and a lower chamber,

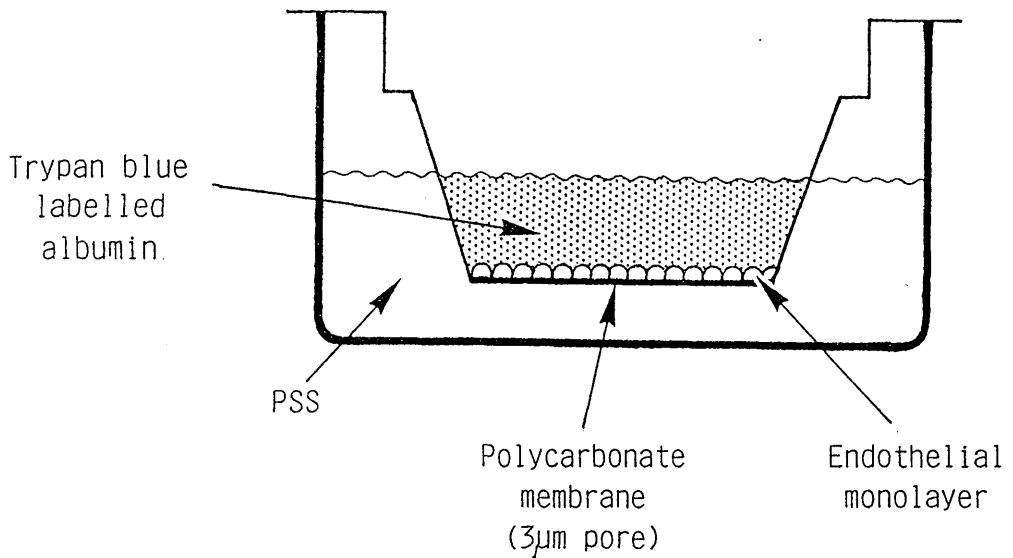


Figure 3.11 Diagrammatic representation of a transwell filter unit. First passage bovine pulmonary artery endothelial cells were cultured on polycarbonate membrane filters (pore size $3\mu\text{M}$) for 3 days. At the end of this period, the lower chamber was filled with physiological salt solution (PSS), typically HEPES (5mM)-buffered Krebs, and the upper chamber with the same buffer but containing trypan blue-labelled albumin (4%). Volumes were chosen which avoided the formation of a hydrostatic gradient and drugs were added to both the upper and lower chambers. The transwells were then agitated on an orbital shaker for 90min and, at the end of this period, a sample was removed from the lower chamber and the transfer of trypan blue-labelled albumin assessed colourimetrically at 590nm.

representing the luminal and abluminal sides of the blood vessel, respectively. Endothelial cells grown on the polycarbonate membrane filter (pore size $3\mu\text{M}$; diameter of filter area 6.5mm) restrict the movement of high molecular weight markers from the upper to the lower compartment. Trypan-blue labelled albumin was used as the high molecular weight marker, although others, such as dextran or ^{125}I -labelled albumin, have also been used by other workers. (Minnear et al., 1989; Stelzner et al., 1989)

3.3.2 Preparation of BPAEC monolayers for permeability studies

Upon reaching confluence, 2 flasks (80cm^2) of BPAEC were washed twice with 20ml of sterile saline and each flask incubated with 10ml of a solution of trypsin (0.05%) and EDTA (0.02%) (Flow laboratories), usually for 2-4 minutes, until all cells had detached. The resultant cell suspension was combined with 2ml of newborn calf serum to inhibit the trypsin. After the cells had been twice spun down (200g; 4 min; 10°C) and washed in 10ml of complete medium, the cells were resuspended in 5ml of complete medium. 1ml of complete medium was added to the lower chamber of each transwell and the accompanying polycarbonate filter placed on top. 100 μl of the suspension of BPAEC was then added to the upper chamber of each transwell (48 filter units in total). The BPAEC were then incubated for 3 days at 37°C in an atmosphere of 95% air/5% CO_2 . At the end of this period, endothelial barrier function was assessed.

3.3.3 Preparation of trypan-blue labelled albumin complex

Trypan blue (180mg) and bovine serum albumin (4g) were dissolved in 100ml of Krebs containing (mM) : NaCl 118, KCl 4.8, MgSO_4 1, KH_2PO_4 1.2, NaHCO_3 2.4, glucose 11 and HEPES 5. This yielded a stable complex with an absorption maximum at 590nm (>99% of the trypan-blue was protein bound, as determined by precipitation with 6% trichloroacetic acid).

3.3.4 Assessment of albumin diffusion across endothelial monolayers

BPAEC monolayers grown on Costar Transwells were washed gently in HEPES(5mM)-buffered Krebs (as used for the preparation of trypan-blue labelled albumin) at 37°C. The monolayers were transferred to a 24 well plate, 600 μ l of HEPES(5mM)-buffered Krebs was placed in the lower chamber and 100 μ l of the trypan-blue labelled albumin (4%) complex in the upper chamber. These volumes were chosen to avoid producing a hydrostatic gradient. Drug additions were made to both the lower and upper chambers. The plate was then incubated at 37°C in air and continuously agitated on an orbital shaker (Luckham) for 90min. At the end of this period, 100 μ l aliquots were sampled from the lower chamber and transferred to plastic cuvettes.

3.3.5 Quantification of albumin diffusion

On completion of an experiment, the 100 μ l lower chamber samples were combined with 900 μ l of HEPES (5mM)-buffered Krebs. The passage of trypan-blue labelled albumin into

the lower chamber was assessed colourimetrically in a dual beam spectrophotometer (Shimadzu) at 590nm and expressed as a percentage of the value that would have been obtained if full equilibration had occurred. 1ml of HEPES-buffered Krebs was used as the reference sample (0% standard), the 100% equilibration standard was prepared by adding 14.3 μ l of trypan-blue labelled albumin (4%) to 985.7 μ l of HEPES(5mM)-buffered Krebs.

3.4 **Materials**

3.4.1 Tissue culture reagents and materials

DMEM, foetal calf serum, newborn calf serum, glutamine and penicillin/streptomycin were all obtained from Gibco, Paisley, UK. HEPES (20mM)-buffered DMEM was obtained from Northumbria Biologicals, Cramlington, UK. Trypsin/EDTA was obtained from Flow laboratories, Irvine, UK., bovine serum albumin (BSA), collagenase (type II) and thymidine were obtained from Sigma, Poole, UK. Collagenase was dissolved in DMEM, yielding a 0.1% solution, and sterilised by filtration through millipore filters (0.2 μ M; Flow laboratories).

A stock solution of thymidine (10^{-2} M) was prepared in distilled water and sterilised by filtration through millipore filters (0.2 μ M; Flow laboratories). Tissue culture flasks and petri dishes were obtained from life technologies, Uxbridge, UK. Costar Transwells were obtained from Northumbria Biologicals, Cramlington, UK.

sterile centrifuge tubes (15ml and 50ml) were obtained from Falcon (UK). Sterile saline (0.9% w/v) was obtained from Baxter Health Care, Thetford, UK. Cylinders of 5% CO₂/95% air were obtained from B.O.C., Ltd. (UK).

3.4.2 Drugs

Adenosine triphosphate (ATP; sodium salt), atriopeptin II, bradykinin triacetate, 8 bromo cyclic 3':5' guanosine monophosphate, caffeine, ethylene glycol (β -aminoethyl ethane) N, N, N', N' - tetracetic acid (EGTA), histamine diphosphate, (\pm) isoprenaline hemisulphate, lipopolysaccharide (E.coli 055:B5), N^G-nitro-L-arginine, 4 α - phorbol 12, 13 didecanoate (4 α - PDD), 4 β - phorbol 12 - myristate 13 - acetate (PMA), sodium nitroprusside, thrombin (bovine) and 3, 4, 5 - trimethoxybenzoic acid 8 - diethylamino acetyl ester (TMB-8) were all obtained from Sigma, Poole, Dorset, UK. Forskolin, fura-2 acid, fura-2 penta-acetoxymethylester (fura-2/AM), ionomycin, pertussis toxin, platelet activating factor and staurosporine were all obtained from Novabiochem, Cambridge, UK. All other reagents were of analytical grade. Stock solution of all drugs were made in distilled water, except for forskolin, fura-2/AM, ionomycin and staurosporine which were dissolved in dimethylsulphoxide (DMSO), 4 α - PDD, PMA and TMB-8 which were dissolved in 100% ethanol and caffeine was dissolved in HEPES(10mM)-buffered Krebs. EGTA was dissolved in distilled water, but 2M sodium hydroxide solution was added to raise the pH for use in some experimental calibrations.

In experiments in which $[Ca^{2+}]_i$ was measured in cells, final cuvette concentrations of ethanol and DMSO did not exceed 0.1%. In experiments in which endothelial barrier function was measured, dilutions of stock solutions were made in HEPES (5mM)-buffered Krebs such that final concentrations of ethanol and DMSO did not exceed 0.06% and 0.1%, respectively.

3.5 Statistical Analysis of Results

Results are expressed as the mean \pm s.e. mean and comparisons were made by Student's t-test or by the Mann-Whitney test when there was unequal variance in the samples. A probability of 0.05 or less was considered significant.

RESULTS

4.1 **Calcium Mobilisation in the Endothelium**

4.1.1 Agonist-induced elevations of $[Ca^{2+}]_i$ in bovine aortic endothelial cells (BAEC)

The ability of the vascular endothelium to play a major role in the regulation of blood vessel tone depends, to a considerable extent, on the release of potent vasodilators such as prostacyclin (Moncada et al., 1976a) and endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980). EDRF release is promoted by a diverse range of mediators, including bradykinin, A23187, substance P, ATP, histamine and thrombin (Furchgott, 1984). As the release of both EDRF and prostacyclin appears to be calcium-dependent (Weksler et al., 1978; Singer & Peach, 1982; Long & Stone, 1985), it is important to consider how endothelial calcium mobilisation may be modulated by intracellular messengers. Consequently, the object of this section was to determine which mediators elevate $[Ca^{2+}]_i$ in cultures of bovine aortic endothelial cells (BAEC) and, subsequently, investigate how other second messenger systems modulate calcium mobilisation.

4.1.2 Effects of bradykinin on $[Ca^{2+}]_i$ in BAEC

In the presence of 1.8mM extracellular calcium, the basal level of $[Ca^{2+}]_i$ in first passage BAEC was 102 ± 3 nM (n=89). Bradykinin induced a biphasic elevation of $[Ca^{2+}]_i$, consisting of a large, transient elevation of $[Ca^{2+}]_i$ which

peaked within 30s, followed by a second component which was relatively well-maintained. This latter component will be termed the plateau phase of the increase in $[Ca^{2+}]_i$ (Figure 4.1). The peak of the initial transient elevation of $[Ca^{2+}]_i$ and the plateau phase of the increase in $[Ca^{2+}]_i$ reached maximum levels of $917 \pm 127 \text{ nM}$ ($n=29$) and $230 \pm 25 \text{ nM}$ ($n=29$), respectively. Both components were concentration-dependent between 0.3 nM and 100 nM ($EC_{50} = 2 \text{ nM}$ for each of the components).

The elevations of $[Ca^{2+}]_i$ induced by bradykinin were, in general, of a biphasic nature; however, in a limited number of experiments (2 out of 400), bradykinin (0.3 nM) induced oscillations of $[Ca^{2+}]_i$ (Figure 4.2). This observation was only made at the lowest concentration of bradykinin examined (0.3 nM).

The elevation of $[Ca^{2+}]_i$ in response to bradykinin (100 nM) was maximal as a second addition of bradykinin (100 nM) produced no further elevation of $[Ca^{2+}]_i$ (Figure 4.3).

Addition of bradykinin (10 nM), to fura-2 loaded BAEC, resulted in an increase in fluorescence following excitation at 340 nm , but not at the calcium-insensitive, isobestic wavelength of 360 nm (Figure 4.4).

4.1.3 Effects of ATP on $[Ca^{2+}]_i$ in BAEC

In the presence of 1.8 mM extracellular calcium, the basal level of $[Ca^{2+}]_i$ in BAEC, in this set of experiments, was

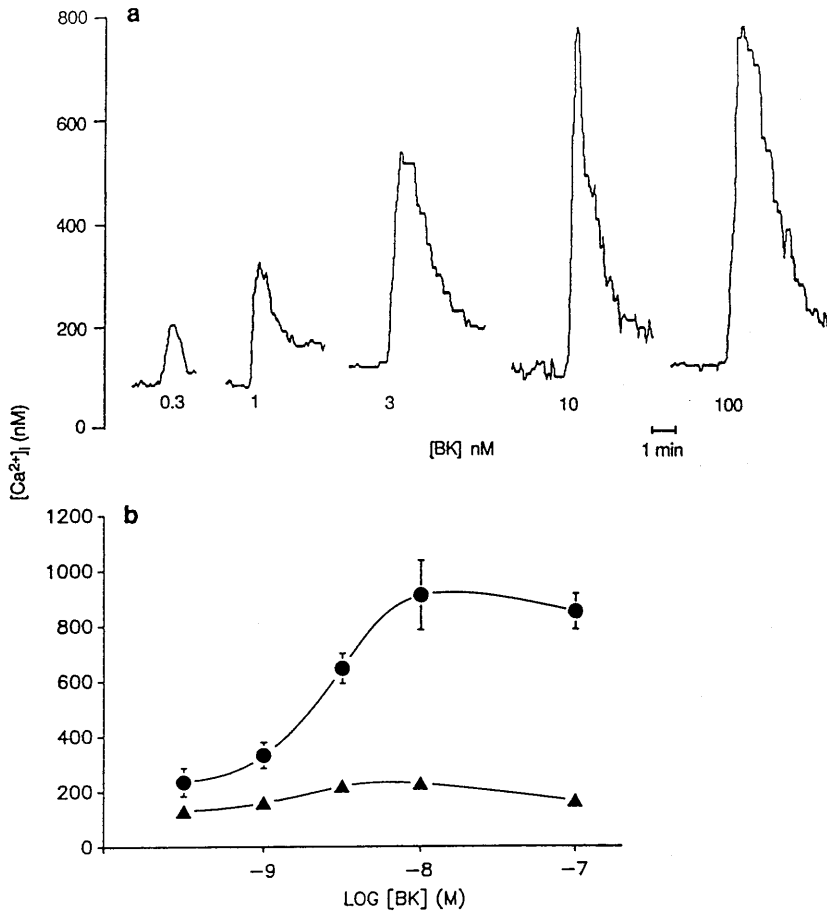


Figure 4.1 Bradykinin (BK, 0.3-100nM) induced a concentration-dependent elevation of $[Ca^{2+}]_i$ in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium. (a) Individual traces showing the biphasic nature of the response to bradykinin : a large, initial transient elevation followed by a lower, more sustained component. (b) Concentration-effect curves show the magnitude of the initial transient increase in $[Ca^{2+}]_i$ (circles) and the sustained phase (triangles) measured 5min after addition of bradykinin. Individual points represent the mean of 8-29 observations and vertical bars indicate the s.e. mean. Where error bars are not seen, they are encompassed within the size of symbols.

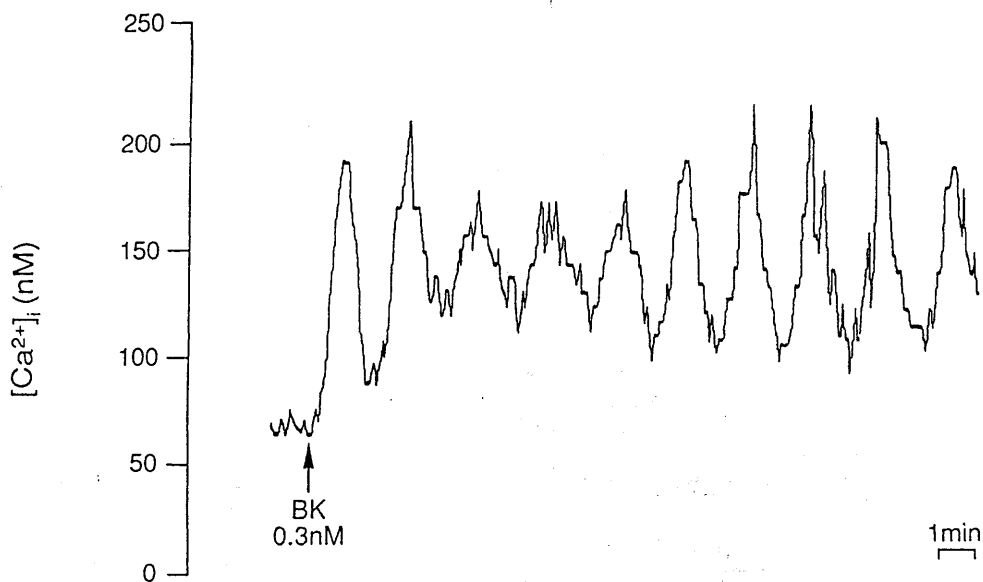


Figure 4.2 At the lowest concentration of bradykinin (BK) examined (0.3nM), a small number of monolayers developed oscillations of $[Ca^{2+}]_i$ subsequent to the addition of bradykinin. The above trace illustrates the characteristic oscillations which were observed in these monolayers of cells.

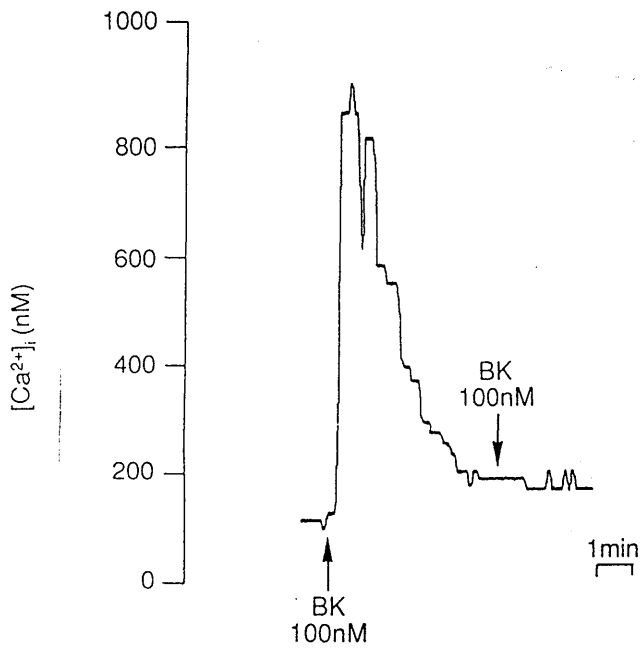


Figure 4.3 In the presence of 1.8mM extracellular calcium, bradykinin (BK; 100nM) induced a biphasic elevation of $[Ca^{2+}]_i$. During the plateau phase of the increase in $[Ca^{2+}]_i$, a subsequent addition of bradykinin (100nM) had no further effect on $[Ca^{2+}]_i$.

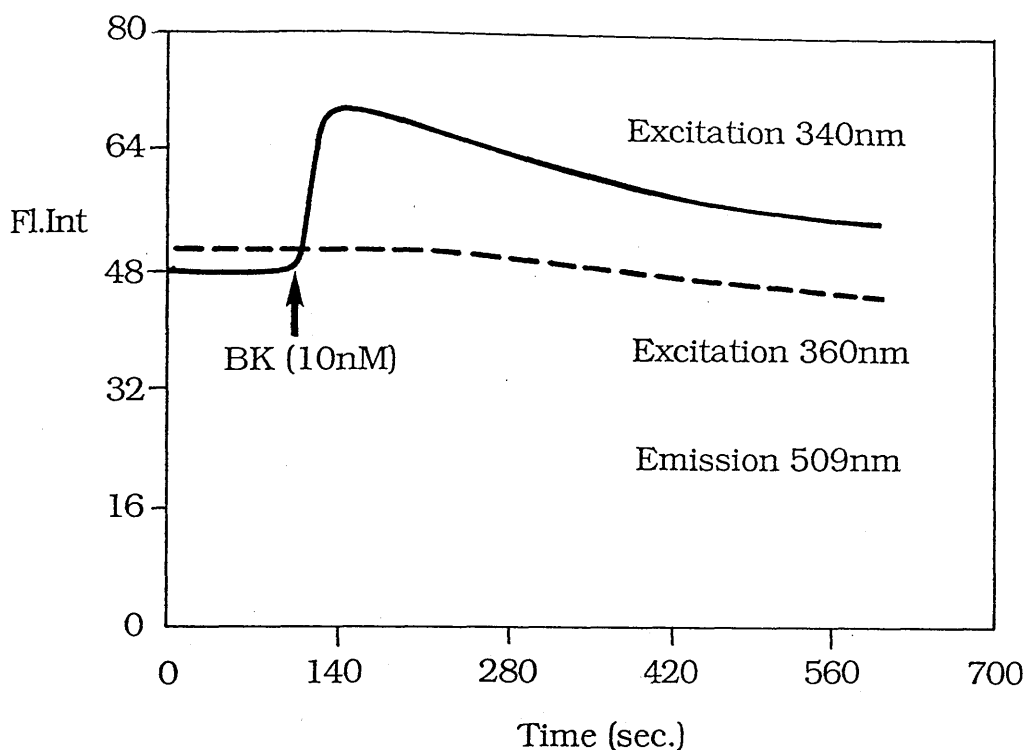


Figure 4.4 Fura-2 loaded bovine aortic endothelial cells were excited alternately at 340nm and 360nm and the resultant fluorescence collected at 509nm. The experimental trace illustrates the change in fluorescence emission following excitation at 340nm (solid line), with addition of bradykinin (BK; 10nM) resulting in an increase in fluorescence emission. In contrast, following excitation at 360nm (broken line) (the isobestic wavelength), addition of bradykinin (10nM) resulted in no change in fluorescence, demonstrating that changes in fluorescence induced by BK only occur at calcium-sensitive wavelengths.

98±3nM (n=120). ATP induced a biphasic elevation of $[Ca^{2+}]_i$ consisting of a large, initial transient component, which peaked within 30s, followed by a well-maintained plateau phase of $[Ca^{2+}]_i$. The peak of the initial transient elevation of $[Ca^{2+}]_i$ and the plateau phase of the increase in $[Ca^{2+}]$ reached maximum levels of 959±2nM (n=44) and 368±16nM (n=44), respectively. Both components were concentration-dependent between 0.3μM and 100μM (Figure 4.5); the EC_{50} for the initial, transient component was 10μM, while the EC_{50} for the plateau phase of the increase in $[Ca^{2+}]_i$ was 6μM.

4.1.4 Effects of thrombin on $[Ca^{2+}]_i$ in BAEC

In the presence of 1.8mM extracellular calcium, the basal level of $[Ca^{2+}]_i$ in BAEC, in this set of experiments, was 91±5nM (n=38). Thrombin induced a biphasic elevation of $[Ca^{2+}]_i$ consisting of a large, initial transient component, which peaked within 30s, followed by a well-maintained plateau phase of $[Ca^{2+}]_i$ (Figure 4.6). The peak of the initial transient elevation of $[Ca^{2+}]_i$ and the plateau phase of the increase in $[Ca^{2+}]_i$ reached maximum levels of 291±30nM (n=20) and 180±13nM (n=20), respectively (Figure 4.6). Both components were concentration-dependent between 0.03U ml⁻¹ and 3U ml⁻¹ (Figure 4.6); the EC_{50} for each component was 0.4U ml⁻¹.

4.1.5 Effects of lipopolysaccharide on $[Ca^{2+}]_i$ in BAEC

Lipopolysaccharide (LPS) has been shown to induce the release of EDRF from BAEC (Salvemini et al., 1989).

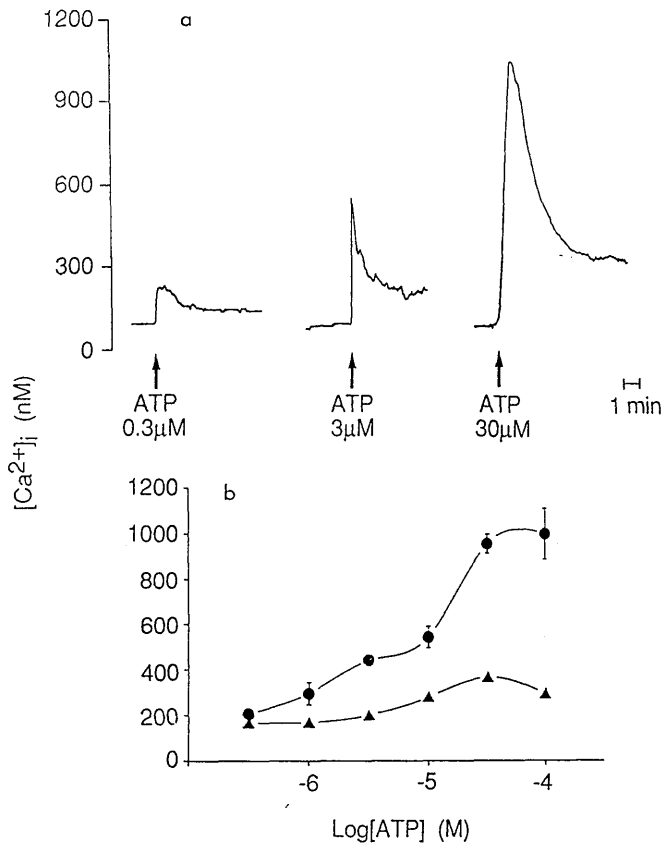


Figure 4.5 ATP (0.3 μ M-100 μ M) induced a concentration-dependent elevation of $[Ca^{2+}]_i$ in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium. (a) Individual traces show the biphasic nature of the response to ATP : a large, initial transient elevation followed by a lower, more sustained component. (b) Concentration-effect curves showing the magnitude of the initial transient increase in $[Ca^{2+}]_i$ (circles) and the sustained phase (triangles) measured 5min after the addition of ATP. Individual points represent the mean of 5-42 observations and vertical bars indicate the s.e. mean. Where error bars are not seen, they are encompassed within the size of the symbols.

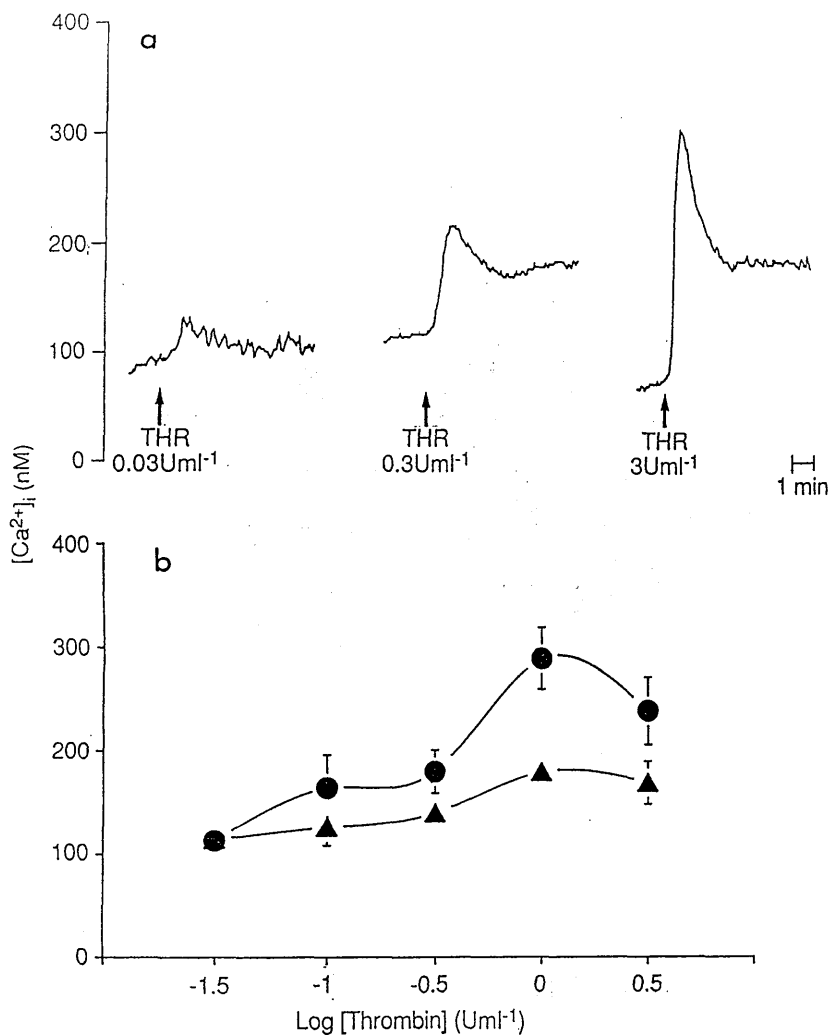


Figure 4.6 Thrombin (THR, 0.03-3U ml⁻¹) induced a concentration-dependent elevation of $[Ca^{2+}]_i$ in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium. (a) Individual traces showing the biphasic nature of the response to thrombin : a large, initial transient elevation followed by a lower, more sustained component. (b) Concentration-effect curves showing the magnitude of the initial transient increase in $[Ca^{2+}]_i$ (circles) and the sustained phase (triangles) measured 5min after addition of thrombin. Individual points represent the mean of 4-20 observations and vertical bars indicate the s.e. mean. Where error bars are not seen, they are encompassed within the size of the symbols.

Addition of LPS ($10\mu\text{g ml}^{-1}$) to BAEC had no effect on $[\text{Ca}^{2+}]_i$ in the presence of 1.8mM extracellular calcium ($n=2$) (Figure 4.7). Cellular responsiveness was confirmed by the addition of thrombin (1U ml^{-1}) which induced a biphasic elevation of $[\text{Ca}^{2+}]_i$.

4.1.6 Effects of histamine on $[\text{Ca}^{2+}]_i$ in BAEC

Histamine ($10\mu\text{M}$) had no effect on $[\text{Ca}^{2+}]_i$ in BAEC ($n=2$) (Figure 4.8). Cellular responsiveness was confirmed by the addition of ATP ($30\mu\text{M}$) which induced a biphasic elevation of $[\text{Ca}^{2+}]_i$.

4.1.7 Effects of platelet activating factor (PAF) on $[\text{Ca}^{2+}]_i$ in BAEC

PAF (120nM) had no effect on $[\text{Ca}^{2+}]_i$ in BAEC in the presence of 1.8mM extracellular calcium ($n=1$) (Figure 4.9). Cellular responsiveness was confirmed by the addition of bradykinin (3nM) which induced a biphasic elevation of $[\text{Ca}^{2+}]_i$.

4.1.8 Effects of ionomycin on $[\text{Ca}^{2+}]_i$ in BAEC

Ionomycin (IONO; 10nM), a divalent cation ionophore (Liu & Herman, 1978), induced a sustained elevation of $[\text{Ca}^{2+}]_i$ in the presence of 1.8mM extracellular calcium ($n=3$) (Figure 4.10a). At a higher concentration ($8\mu\text{M}$), ionomycin increased $[\text{Ca}^{2+}]_i$ to levels beyond the upper range of measurement with fura-2 (approximately $3\mu\text{M}$) (Figure 4.10b).

4.2 **Calcium Mobilisation from Intracellular and**

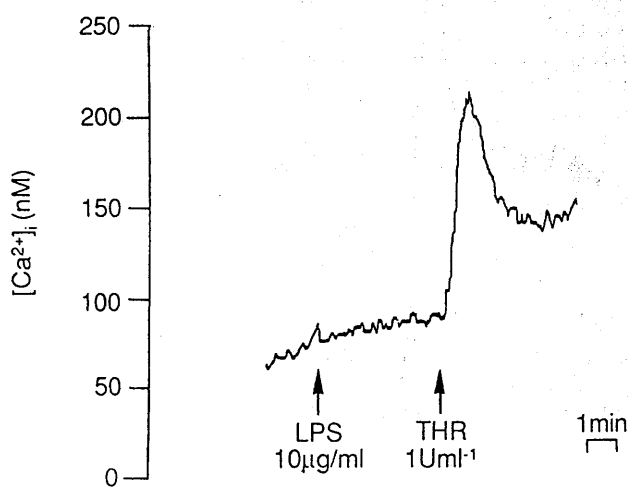


Figure 4.7 Addition of lipopolysaccharide (LPS; 10µg ml⁻¹) to bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium, had no effect on $[Ca^{2+}]_i$. Cellular responsiveness was confirmed by the addition of thrombin (THR; 1U ml⁻¹), which induced a large, biphasic elevation of $[Ca^{2+}]_i$. The lack of effect of LPS on $[Ca^{2+}]_i$, illustrated in the above trace, was obtained in 2 separate experiments.

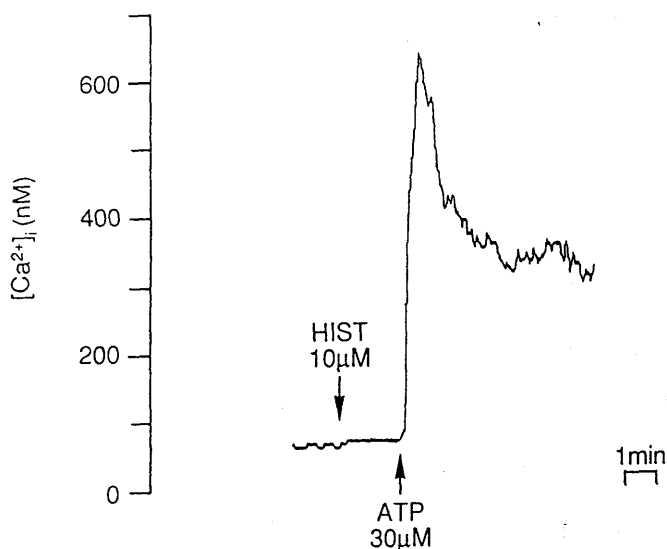


Figure 4.8 Addition of histamine (HIST; $10\mu\text{M}$) to bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium, had no effect on $[\text{Ca}^{2+}]_i$. Cellular responsiveness was confirmed by the addition of ATP ($30\mu\text{M}$) which induced a large, biphasic elevation of $[\text{Ca}^{2+}]_i$. The lack of effect of histamine on $[\text{Ca}^{2+}]_i$ illustrated in the above trace was obtained in 2 separate experiments.

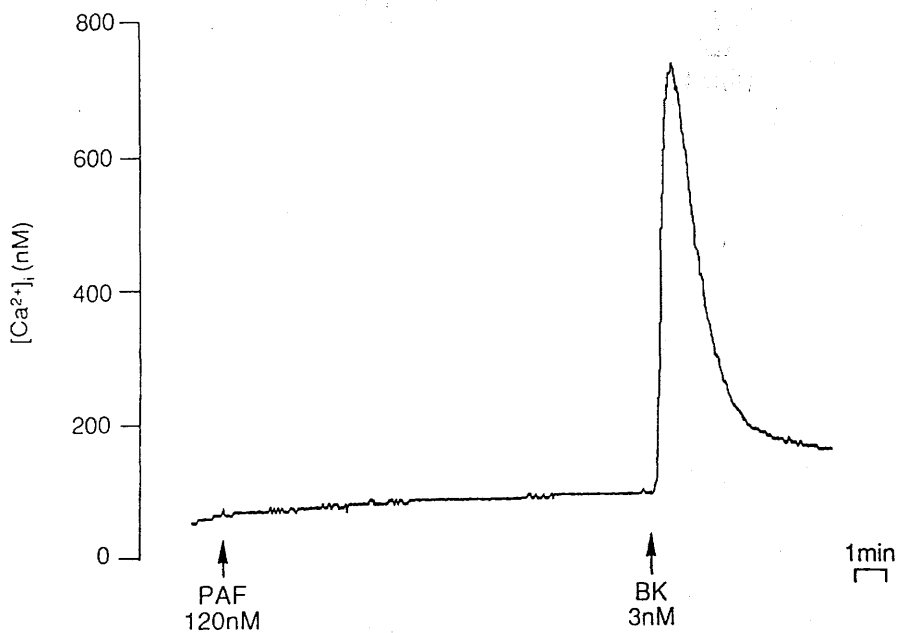


Figure 4.9 Addition of platelet activating factor (PAF; 120nM) to bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium, had no effect on $[Ca^{2+}]_i$. Cellular responsiveness was confirmed by the addition of bradykinin (BK; 3nM), which induced a large biphasic elevation of $[Ca^{2+}]_i$. The effects of PAF were examined only once.

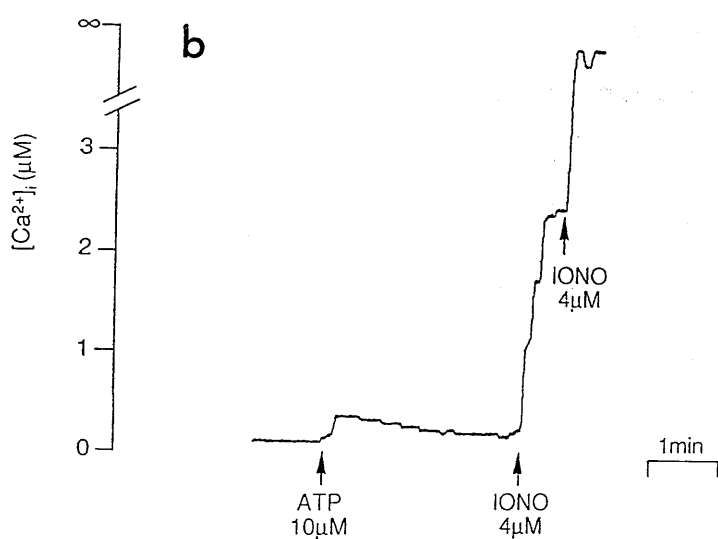
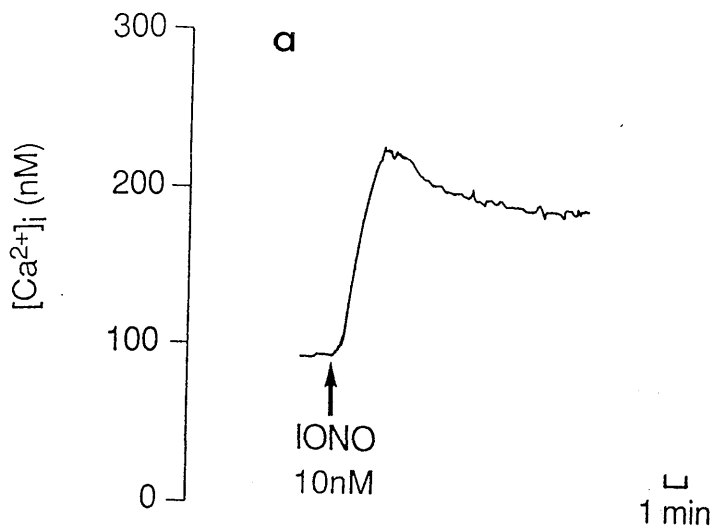


Figure 4.10 Individual traces illustrating the effects of ionomycin (IONO) on $[Ca^{2+}]_i$ in bovine aortic endothelial cells. Traces (a) and (b) illustrate the effects of ionomycin (10 nM and 8 μM, respectively) on $[Ca^{2+}]_i$ in the presence of 1.8 mM extracellular calcium. In trace (b), the concentration of ionomycin (8 μM) added is sufficient to saturate the intracellular fura-2 and, therefore, peak $[Ca^{2+}]_i$ is denoted as infinity. Traces (a) and (b) are each representative of 3 and 4 individual experiments, respectively.

Extracellular Pools

4.2.1 Agonist-induced elevations of $[Ca^{2+}]_i$ in BAEC - role of intracellular and extracellular calcium pools

Agonist-induced elevations of $[Ca^{2+}]_i$ may be derived from 2 calcium pools - the intracellular and the extracellular calcium pools. To distinguish between the release of calcium from intracellular stores and calcium influx from an extracellular pool, two main strategies may be employed; firstly, removal of extracellular calcium or chelation of extracellular calcium using EGTA or, secondly, blockade of calcium influx using nickel (Ni^{2+} ; Hallam et al., 1988b). Endothelial cells have no voltage-operated calcium channels (Hallam & Pearson, 1986; Colden - Stanfield et al., 1987) and, therefore, calcium influx cannot be blocked using organic calcium antagonists.

4.2.2 Effects of nickel on bradykinin-induced elevations of $[Ca^{2+}]_i$ in BAEC

In human umbilical vein endothelial cells, nickel (Ni^{2+}) has been previously shown to block calcium influx (Hallam et al., 1988b). The use of Ni^{2+} , therefore, enables the relative contribution of mobilisation from intracellular and extracellular calcium pools to be assessed.

In the presence of 1.8mM extracellular calcium, treatment with nickel chloride (4mM, 2min) had no effect on basal $[Ca^{2+}]_i$ or on the magnitude of the initial transient

elevation of $[Ca^{2+}]_i$ induced by bradykinin (0.3nM, 10nM) but abolished the plateau phase of the increase in $[Ca^{2+}]_i$ induced by bradykinin (Figure 4.11). Addition of the nickel chloride (4mM) during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by bradykinin (10nM) resulted in the abolition of this component, with $[Ca^{2+}]_i$ falling rapidly to the original basal level (Figure 4.12).

4.2.3 Effects of lowering extracellular calcium concentration on $[Ca^{2+}]_i$ in BAEC

Incubation of BAEC in nominally calcium-free HEPES (10mM)-buffered Krebs for 25 min, followed by addition of EGTA (0.5mM) for 5 min, reduced extracellular calcium to 12 ± 1 nM (n=4) and basal levels of $[Ca^{2+}]_i$ from 88 ± 6 nM (n=11) to 57 ± 8 nM (n=16). Re-addition of calcium (2.3mM) restored $[Ca^{2+}]_i$ to 98 ± 5 nM (n=5). Figure 4.13 illustrates a typical example of the change in basal $[Ca^{2+}]_i$ obtained upon addition of EGTA and the re-addition of extracellular calcium.

In nominally calcium-free Krebs, in the presence of EGTA (0.5mM), the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by bradykinin (10nM) was reduced and the plateau phase of the increase in $[Ca^{2+}]_i$ was abolished (Figure 4.14). In the continued presence of bradykinin, the re-addition of extracellular calcium (2.3mM) induced a biphasic elevation of $[Ca^{2+}]_i$ consisting of a large, transient component, followed by a lower, sustained component (Figure 4.14).

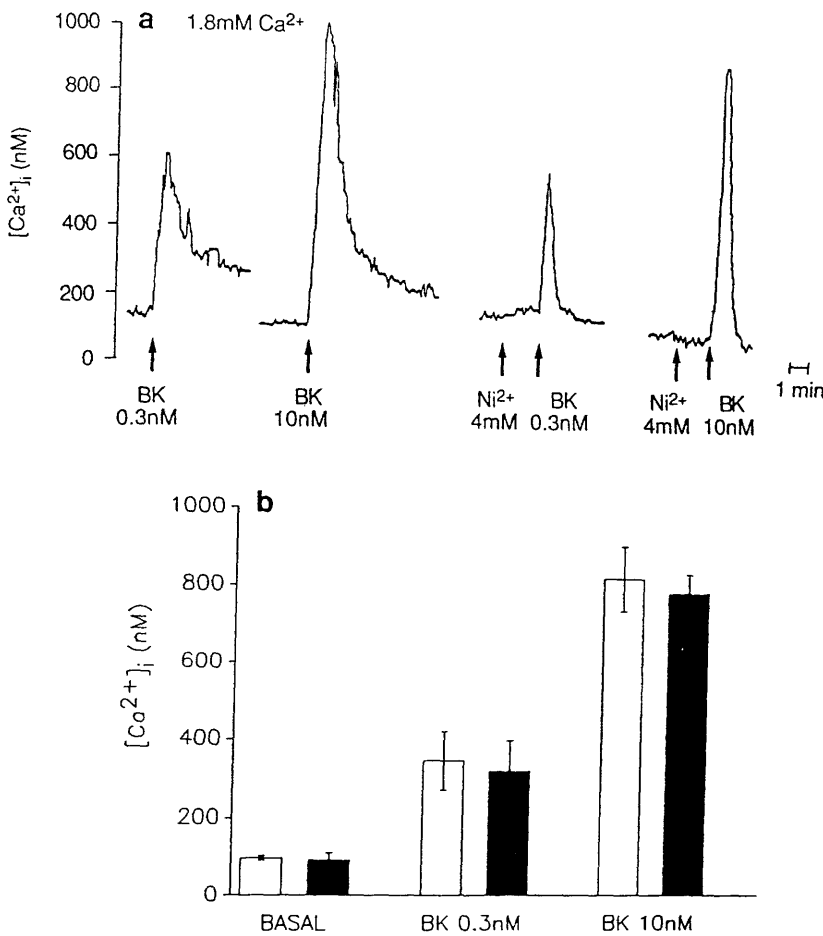


Figure 4.11 Individual traces (a) and a histogram (b) showing basal and bradykinin (BK; 0.3nM and 10nM) - stimulated levels of $[Ca^{2+}]_i$ in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium (open columns) and following pre-treatment with nickel (Ni^{2+} ; 4mM, 2min, solid columns). In the histogram, basal levels and the magnitude of the initial transient elevations of $[Ca^{2+}]_i$ are given as the mean of 4-19 observations and vertical bars indicate the s.e. mean.

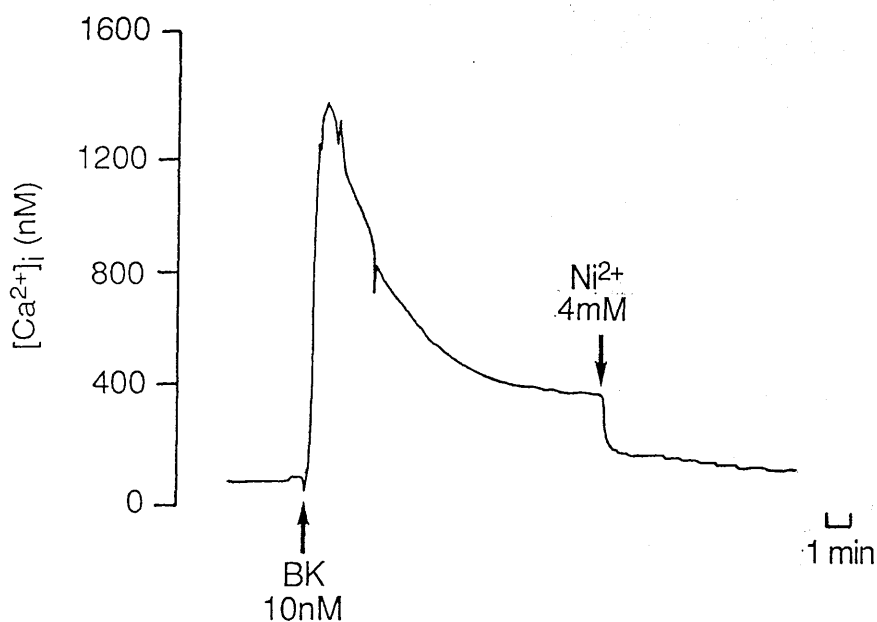


Figure 4.12 In the presence of 1.8mM extracellular calcium, bradykinin (BK; 10nM) induced a biphasic elevation of $[Ca^{2+}]_i$, consisting of a large, initial transient elevation of $[Ca^{2+}]_i$, followed by a more sustained component (the plateau phase of the increase in $[Ca^{2+}]_i$). Addition of nickel (Ni^{2+} ; 4mM) during the plateau phase of the increase in $[Ca^{2+}]_i$ resulted in a rapid fall in $[Ca^{2+}]_i$ to near basal levels.

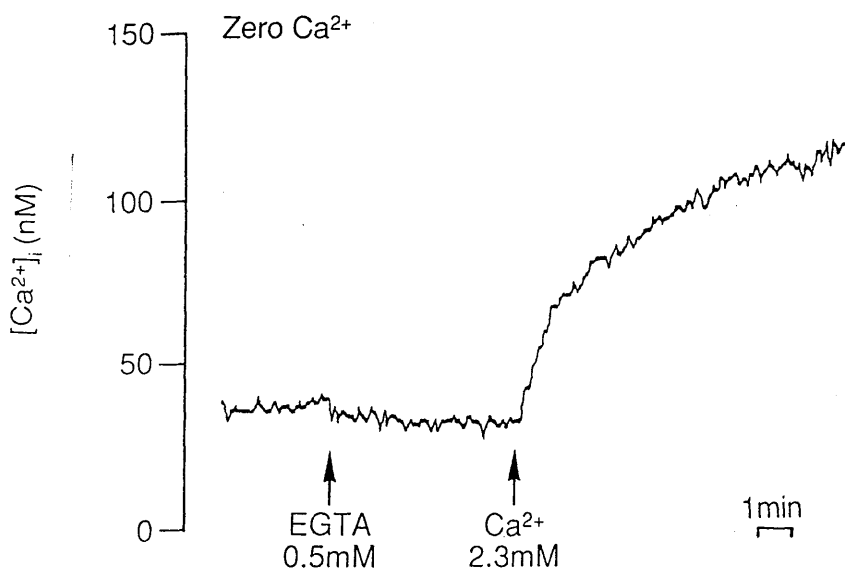


Figure 4.13 Bovine aortic endothelial cells were incubated in nominally calcium-free HEPES (10mM)-buffered Krebs for 25 min, followed by addition of EGTA (0.5mM) for 5 min. The subsequent addition of extracellular calcium (2.3mM) resulted in $[Ca^{2+}]_i$ rising to a new, sustained level. The above experimental trace is typical of that obtained in 5 separate experiments.

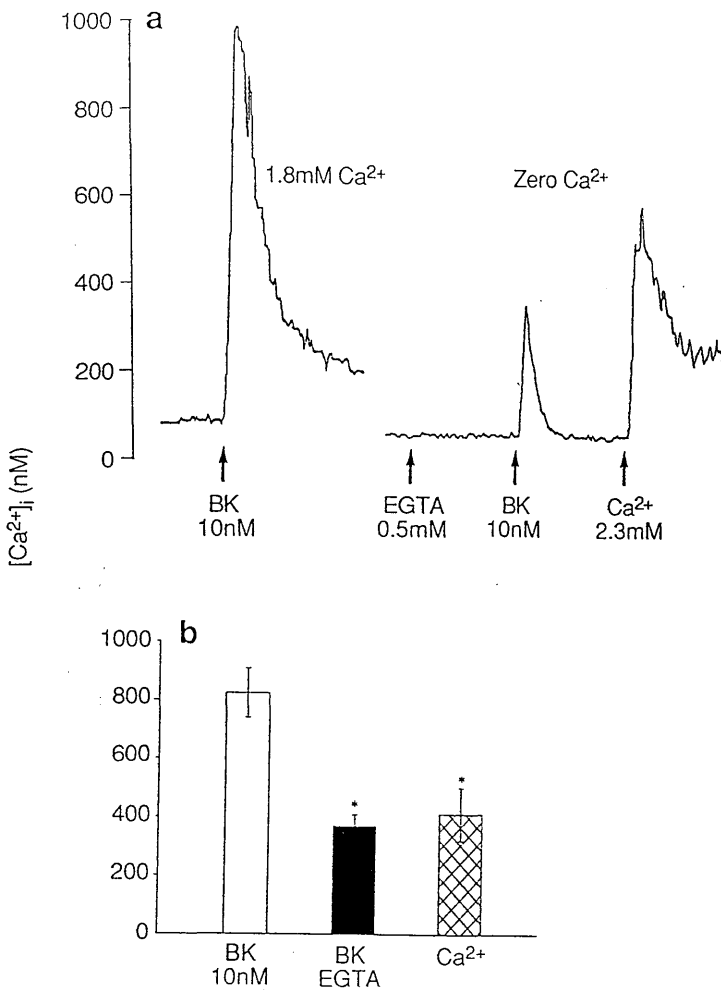


Figure 4.14 Individual traces (a) and a histogram (b) illustrating the effects of bradykinin (BK; 10nM) on $[Ca^{2+}]_i$ in bovine aortic endothelial cells, in the presence of 1.8mM calcium (open column), and following the removal of extracellular calcium and addition of EGTA (0.5mM, 5min, solid column). The effect of re-addition of calcium (Ca^{2+} ; 2.3mM) in the continued presence of bradykinin is shown in the cross-hatched columns. In the histogram, the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ is the mean of 6 observations and vertical bars indicate the s.e. mean. *p<0.05, indicates a significant difference from the response to bradykinin in the presence of calcium.

Incubation of BAEC in nominally calcium-free Krebs containing EGTA (0.5mM) almost completely abolished the initial transient elevation of $[Ca^{2+}]_i$ induced by bradykinin (1nM) and abolished the plateau phase of the increase in $[Ca^{2+}]_i$ (Figure 4.15). Subsequent re-addition of extracellular calcium (2.3mM) induced a biphasic elevation of $[Ca^{2+}]_i$, the magnitude of both components being similar to those induced by bradykinin (1nM) in the presence of 1.8mM extracellular calcium (Figure 4.15).

Incubation of BAEC in nominally calcium-free Krebs containing EGTA (0.5mM) attenuated the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by ATP (100 μ M) and abolished the plateau phase of the increase in $[Ca^{2+}]_i$ (Figure 4.16). In the continued presence of ATP (100 μ M), re-addition of extracellular calcium (2.3mM) induced a biphasic elevation of $[Ca^{2+}]_i$, consisting of a large, transient component followed by a lower, more sustained component (Figure 4.16).

In an attempt to determine the kinetics of the loss of the bradykinin-induced transient elevation of $[Ca^{2+}]_i$ in BAEC, which occurs in the absence of extracellular calcium, we examined the effects of the acute addition of EGTA for different times and at different concentrations. All experiments were carried out in HEPES (10mM)-buffered Krebs containing 1mM calcium chloride. In this buffer, bradykinin (10nM) induced similar elevations of $[Ca^{2+}]_i$ to those obtained in 1.8mM calcium (Figures 4.1 and 4.17). In

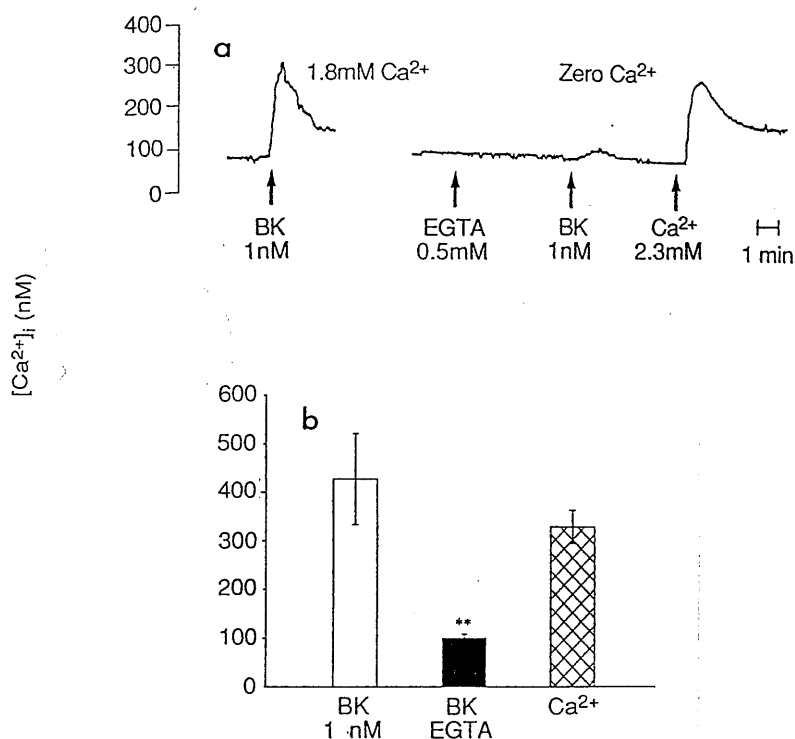


Figure 4.15 Individual traces (a) and histogram (b) illustrating the effects of bradykinin (BK; 1nM) on $[Ca^{2+}]_i$ in bovine aortic endothelial cells, in the presence of 1.8mM calcium (open columns) and following the removal of extracellular calcium and addition of EGTA (0.5mM, 5min, solid column). The effect of re-addition of calcium (Ca^{2+} ; 2.3mM) in the continued presence of bradykinin is shown in the cross-hatched column. In the histogram, the magnitudes of the initial transient elevation of $[Ca^{2+}]_i$ are the mean of 5-7 observations and vertical bars indicate the s.e. mean. ** $p < 0.01$, indicates a significant difference from the response to bradykinin in the presence of calcium.

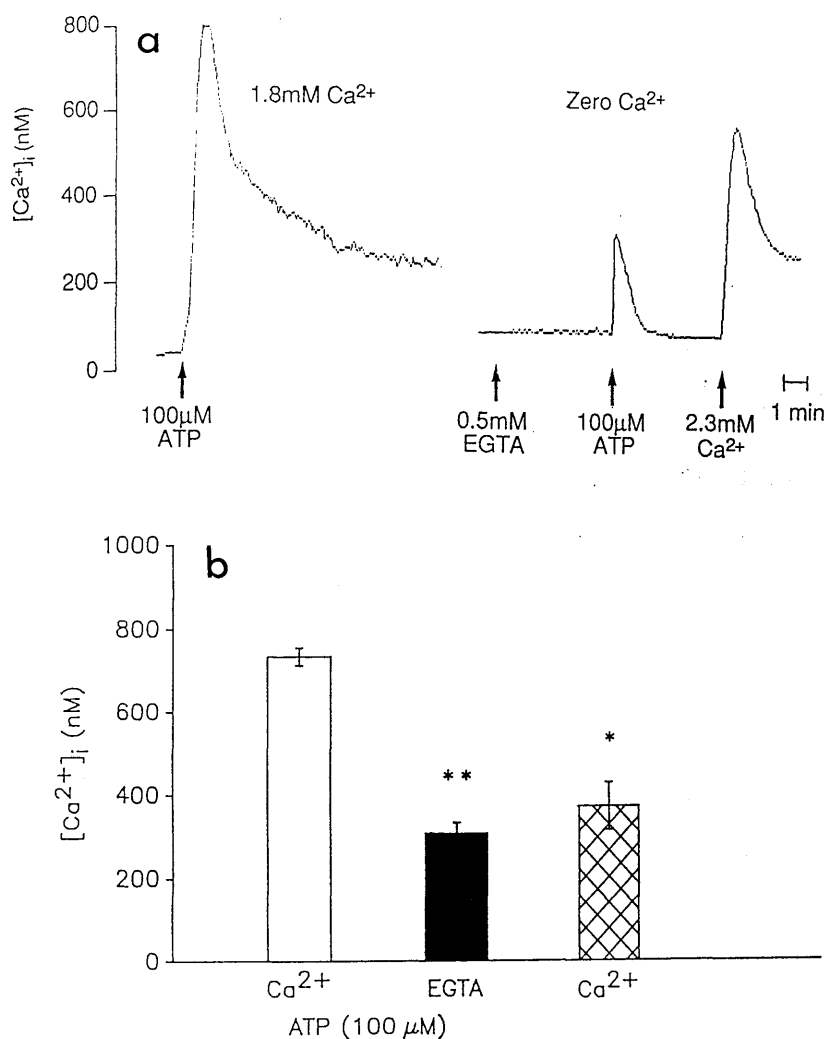


Figure 4.16 Individual traces (a) and histogram (b) illustrating the effect of ATP (100 μ M) on $[Ca^{2+}]_i$ in bovine aortic endothelial cells, in the presence of 1.8mM calcium (open column) and following the removal of extracellular calcium and addition of EGTA (0.5mM, 5min, solid column). The effect of re-addition of calcium (Ca^{2+} ; 2.3mM) in the continued presence of ATP is shown in the cross-hatched column. In the histogram, the magnitudes of the initial transient elevation of $[Ca^{2+}]_i$ are the mean of 6 observations and vertical bars indicate the s.e. mean. ** $p < 0.01$, indicates a significant difference from the response to ATP in the presence of calcium.

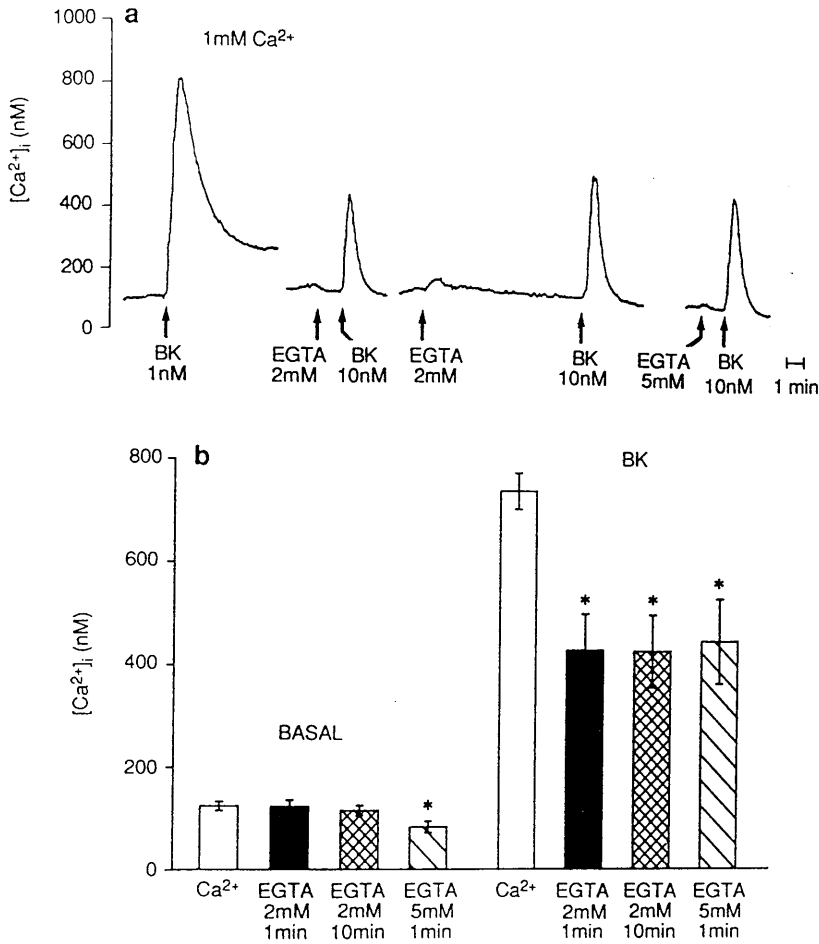


Figure 4.17 Individual traces (a) and histogram (b) showing the effects of pre-treatment with EGTA (2mM and 5mM) for 1 min or 10 min on basal and bradykinin (10nM) - stimulated levels of [Ca²⁺]_i in bovine aortic endothelial cells, in the presence of 1mM extracellular calcium. In the histogram, basal levels and the magnitudes of the initial transient elevation are given as the mean of 4-16 observations and vertical bars indicate the s.e. mean. *p<0.05 indicates a significant difference from values obtained in the presence of 1mM extracellular calcium.

the presence of 1mM extracellular calcium, addition of EGTA (2mM) for 1 min lowered extracellular calcium to $560 \pm 30 \text{ nM}$, slightly reduced basal $[\text{Ca}^{2+}]_i$ (although not significantly), reduced the magnitude of the initial transient elevation of $[\text{Ca}^{2+}]_i$ induced by bradykinin (10nM) and abolished the plateau phase of the increase in $[\text{Ca}^{2+}]_i$ (Figure 4.17). Exposure of BAEC to EGTA (2mM) for 10 min had no effect on resting $[\text{Ca}^{2+}]_i$ and produced no further reduction in the magnitude of the bradykinin (10nM)-induced transient elevation of $[\text{Ca}^{2+}]_i$ than that obtained after 1 min exposure (Figure 4.17). Exposure to a higher concentration of EGTA (5mM) for 1 min lowered extracellular calcium to $101 \pm 2 \text{ nM}$ ($n=4$) and induced a small, but significant, fall in basal $[\text{Ca}^{2+}]_i$ (Figure 4.17). However, this higher concentration of EGTA produced no further reduction in the magnitude of the bradykinin (10nM)-induced transient elevation of $[\text{Ca}^{2+}]_i$ than that obtained with a concentration of 2mM (Figure 4.17). The effects of higher concentrations of EGTA could not be examined since this treatment leads to significant detachment of cells from the coverslip.

In BAEC bathed in HEPES(10mM)-buffered Krebs containing CaCl_2 (1mM) and EGTA (2mM), bradykinin (10nM) induced a transient elevation of $[\text{Ca}^{2+}]_i$, which returned to basal levels within 100s (Figure 4.18). Subsequent addition of ionomycin ($1 \mu\text{M}$) induced a transient elevation of $[\text{Ca}^{2+}]_i$ which returned to near basal levels within 200s (Figure

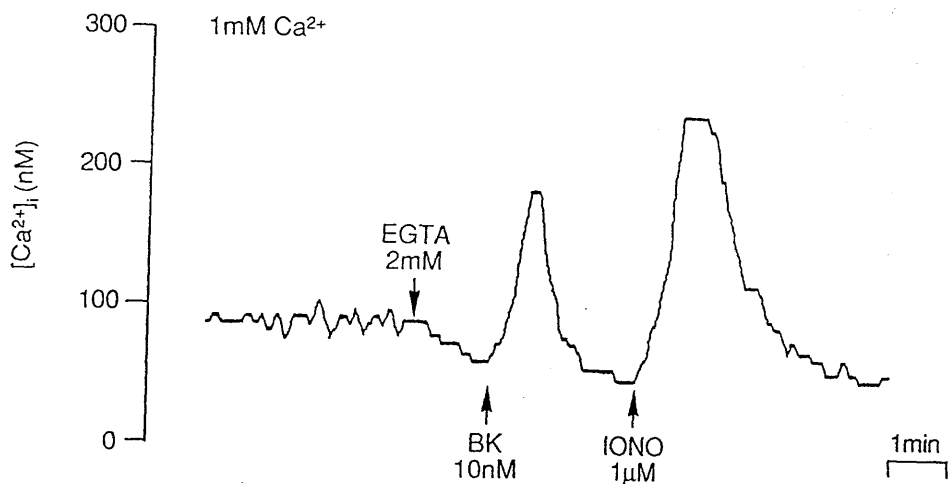


Figure 4.18 An individual trace showing the effects of bradykinin (BK; 10nM) and ionomycin (IONO; 1 μ M) on $[Ca^{2+}]_i$ in bovine aortic endothelial cells, in the absence of extracellular calcium. Chelation of extracellular calcium with EGTA (2mM) resulted in a small fall in basal $[Ca^{2+}]_i$. Addition of bradykinin (10nM) induced a transient elevation of $[Ca^{2+}]_i$ which returned to basal levels within 100s. Subsequent addition of ionomycin (1 μ M) induced a transient elevation of $[Ca^{2+}]_i$ which returned to basal values within 200s. This trace is representative of 2 experimental traces.

4.18). Therefore, ionomycin may release calcium from intracellular as well as extracellular stores.

4.2.4 Effects of caffeine on $[Ca^{2+}]_i$ in BAEC

The possibility that removal of extracellular calcium depleted intracellular stores of calcium was tested further by examining the effects of caffeine, which is known to promote the release of calcium from these stores (Weber & Herz, 1968). In the presence of 1.8mM extracellular calcium, caffeine (5mM) induced a small elevation of $[Ca^{2+}]_i$ (Figure 4.19). Following incubation in nominally calcium-free Krebs for 25 min, followed by the addition of EGTA (0.5mM) for 5 min, basal $[Ca^{2+}]_i$ was reduced significantly and the ability of caffeine (5mM) to elevate $[Ca^{2+}]_i$ was abolished (Figure 4.19).

4.2.5 Effects of TMB-8 on bradykinin-induced elevations of $[Ca^{2+}]_i$ in BAEC

In the presence of 1.8mM extracellular calcium, treatment with TMB-8 (0.1mM), a putative inhibitor of intracellular calcium release (Malagodi & Chiou, 1974), induced a small elevation of $[Ca^{2+}]_i$ (58 ± 6 nM; $n=4$), but had no effect on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by bradykinin (3nM) (Figure 4.20).

A more careful analysis of the effects of TMB-8 on the release of calcium from intracellular stores was performed in nominally calcium-free HEPES(10mM)-buffered Krebs containing EGTA (0.5mM), so as to remove the calcium influx

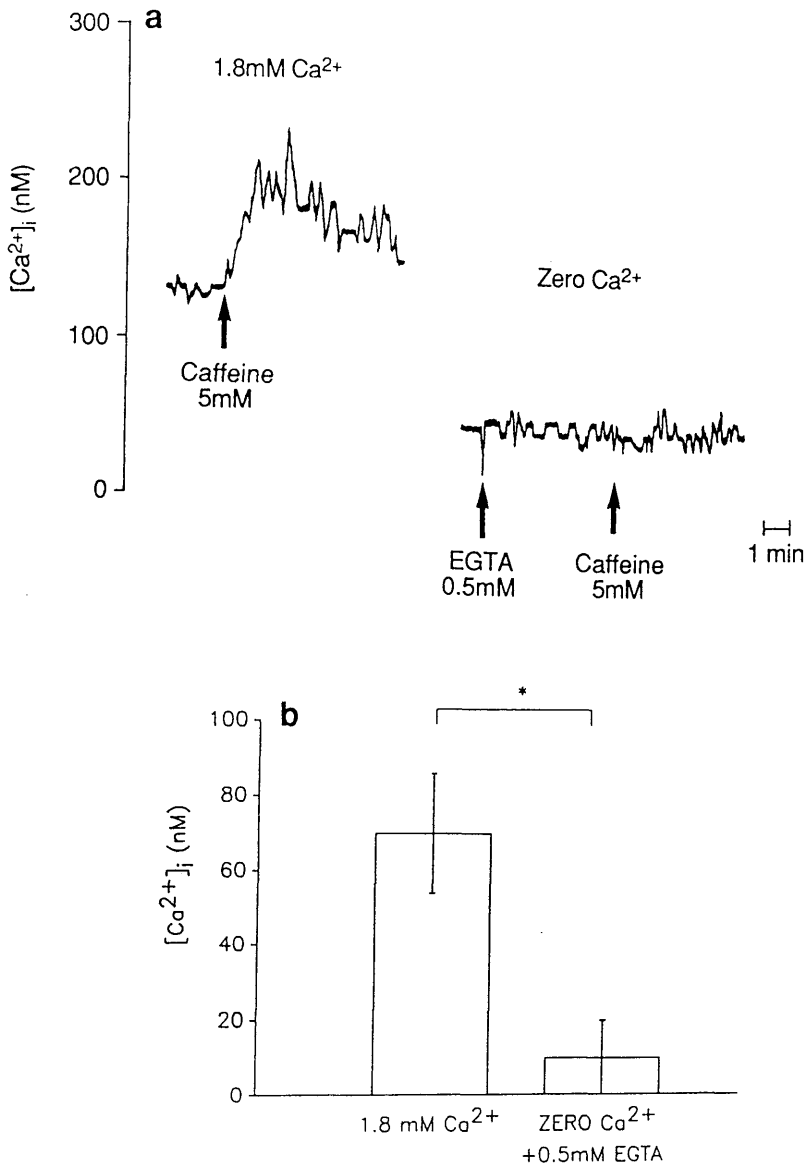


Figure 4.19 Individual traces (a) and histogram (b) showing the effects of removal of extracellular calcium and addition of EGTA (0.5mM, 5min) on caffeine-stimulated levels of $[\text{Ca}^{2+}]_i$ in bovine aortic endothelial cells. In the histogram, the magnitude of the elevations of $[\text{Ca}^{2+}]_i$ induced by caffeine in the absence and presence of 1.8mM extracellular calcium are the mean of 5-7 observations and vertical bars indicate the s.e. mean. *p<0.05 indicates a significant difference between groups joined by a bracket.

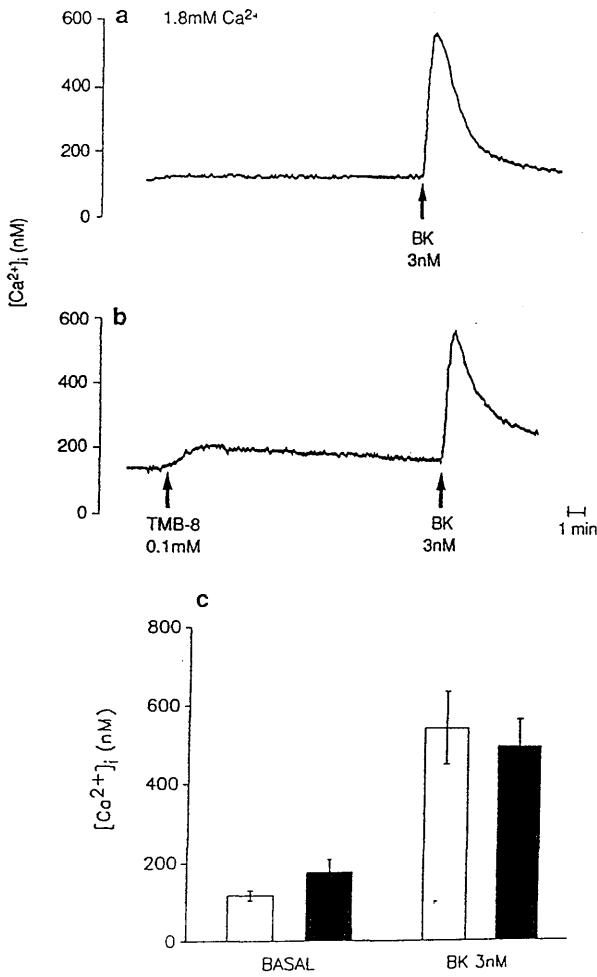


Figure 4.20 Individual traces (a and b) and a histogram (c) showing basal and bradykinin (BK; 3nM) - stimulated levels of $[Ca^{2+}]_i$ in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium (open columns) and following treatment with 3,4,5-trimethoxybenzoic acid 8-(diethylamino) octyl ester (TMB-8; 0.1mM, 15min, solid columns). In the histogram, basal levels and the magnitude of the initial transient elevations of $[Ca^{2+}]_i$ are the mean of 4-8 observations and vertical bars indicate the s.e. mean.

component. Even under these conditions, TMB-8 (0.1mM, 3min) had no effect on the transient elevation of $[Ca^{2+}]_i$ induced by bradykinin (10nM) (Figure 4.21). In the continued presence of bradykinin (10nM), re-addition of extracellular calcium (2.3mM) induced a biphasic elevation of $[Ca^{2+}]_i$ and this was also completely unaffected in the presence of TMB-8 (Figure 4.21).

4.2.6 Effects of procaine on bradykinin-induced mobilisation of $[Ca^{2+}]_i$

Following incubation in nominally calcium-free HEPES (10mM)-buffered Krebs for 25 min, followed by EGTA (0.5mM) for 5 min, bradykinin (10nM) induced a transient elevation of $[Ca^{2+}]_i$ which returned close to basal levels within 90s (Figure 4.22). Subsequent addition of procaine (1mM), an inhibitor of calcium-induced calcium release (Weber & Herz, 1968; Saida & van Breemen, 1984), induced an apparent fall in $[Ca^{2+}]_i$ (Figure 4.22). However, procaine (1mM) was found to reduce fura-2 fluorescence between excitation wavelengths of 300nm and 350nm (Figure 4.23); this selective reduction in fluorescence at 340nm, but not 380nm, is likely to account for the apparent fall in $[Ca^{2+}]_i$. In the continued presence of bradykinin (10nM) and procaine (1mM), re-addition of extracellular calcium (2.3mM) yielded a biphasic elevation of $[Ca^{2+}]_i$. The inability of procaine to abolish the transient elevation obtained upon re-addition of extracellular calcium, suggests that this component is not dependent upon calcium-induced calcium release.

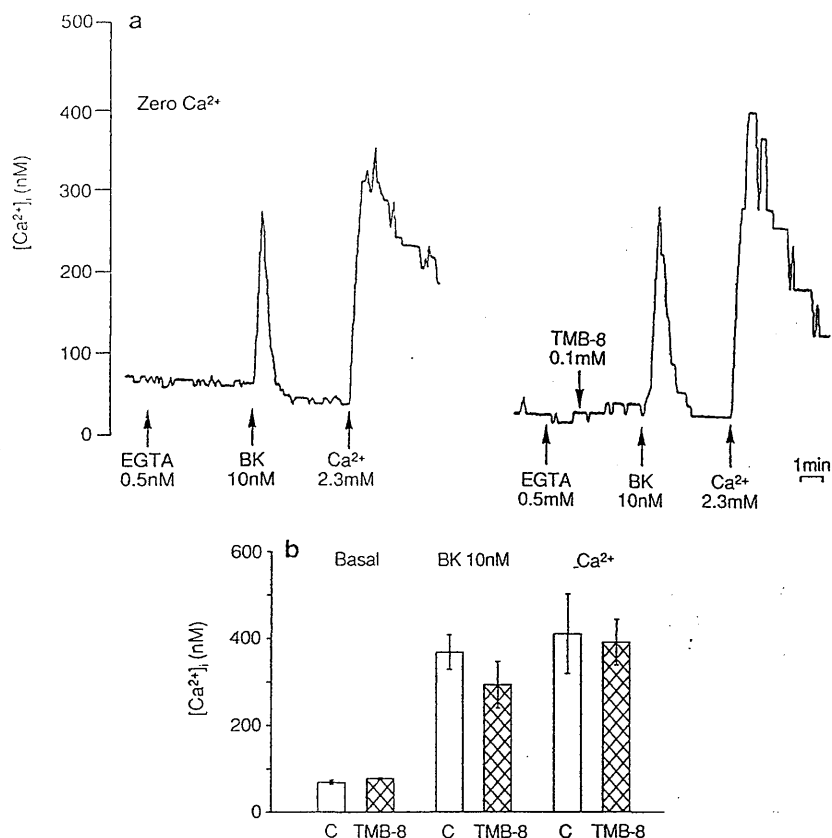


Figure 4.21 Bovine aortic endothelial cells were incubated in nominally calcium-free Krebs for 25 min followed by EGTA (0.5 mM) for a further 5 min. The individual traces (a) and histogram (b) illustrate the effects of pre-treatment with TMB-8 (0.1 mM, 3 min) on the transient elevation of $[Ca^{2+}]_i$ induced by bradykinin (BK; 10 nM) and the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ obtained upon subsequent re-addition of extracellular calcium (2.3 mM), in the continued presence of bradykinin. In the histogram, levels of $[Ca^{2+}]_i$ in control cells (open columns) and in TMB-8 cells (cross-hatched columns) are the mean of 4-10 observations and vertical bars indicate the s.e. mean.

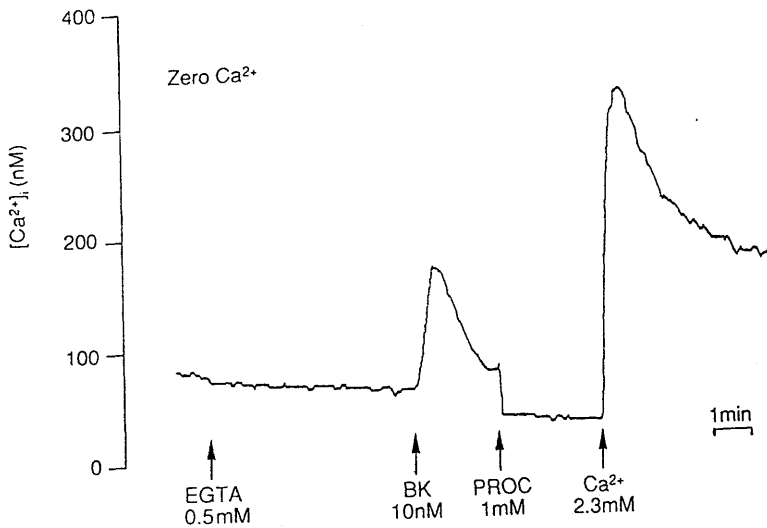


Figure 4.22 Monolayers of bovine aortic endothelial cells were incubated in nominally calcium-free HEPES-buffered Krebs for 25 min and EGTA (0.5mM) was then added for 5 min. Addition of bradykinin (BK; 10nM) then induced a transient elevation of $[Ca^{2+}]_i$ and subsequent addition of procaine (PROC; 1mM) resulted in an apparent fall in $[Ca^{2+}]_i$. In the continued presence of bradykinin and procaine, addition of extracellular calcium (2.3mM) induced a biphasic elevation of $[Ca^{2+}]_i$.

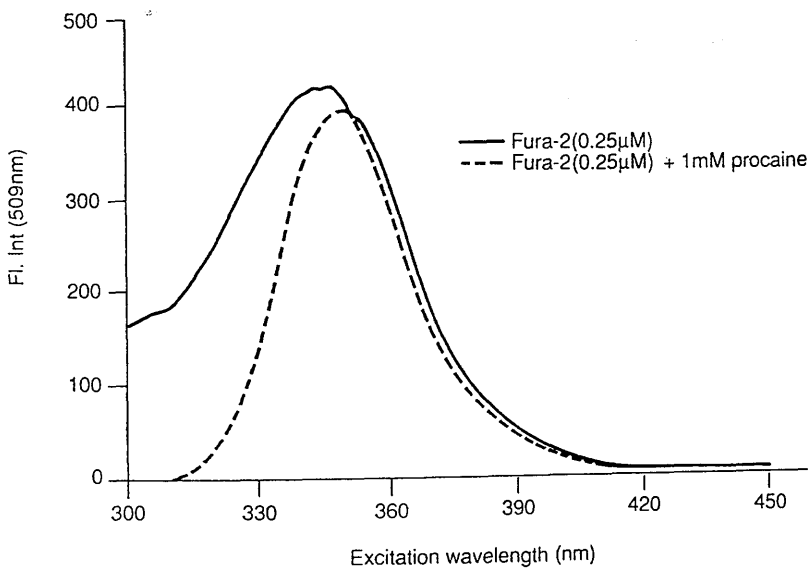


Figure 4.23 Excitation spectra of fura-2 ($0.25\mu\text{M}$) in HEPES-buffered Krebs containing 1.8mM calcium chloride, in the presence (broken line) and absence (solid line) of procaine (1mM).

4.3 **Effects of Membrane Depolarisation**

4.3.1 Effects of membrane depolarisation on $[Ca^{2+}]_i$ in BAEC

In both vascular and non-vascular smooth muscle, membrane depolarisation, induced by high potassium-containing solutions, results in increased calcium entry via voltage-operated channels (VOCs) and the subsequent generation of muscle contraction (Bolton, 1979). As already discussed, calcium entry in endothelial cells does not occur via VOCs (Hallam & Pearson, 1986), but more recent studies have suggested that changes in membrane potential may modulate $[Ca^{2+}]_i$ in atrial endocardial endothelial cells (Laskey et al., 1990). To determine if changes in membrane potential could modulate mobilisation of $[Ca^{2+}]_i$ in bovine aortic endothelial cells, we examined the effects of solutions containing high concentrations of potassium on basal and bradykinin-simulated levels of $[Ca^{2+}]_i$.

4.3.2 Effects of potassium chloride on $[Ca^{2+}]_i$ in BAEC

In the presence of 1.8mM extracellular calcium, addition of potassium chloride (KCl; 30mM or 60mM, 1 min) to normal, isotonic Krebs had no effect on basal $[Ca^{2+}]_i$ in BAEC (Figures 4.24 and 4.25). Furthermore, pre-treatment with KCl (30mM, 1 min) had no effect on the magnitude of the bradykinin (10nM)-induced elevation of $[Ca^{2+}]_i$ (Figure 4.24). In contrast, pre-treatment with KCl (60mM, 1 min) significantly inhibited the magnitude of the bradykinin (10nM)-induced transient elevation of $[Ca^{2+}]_i$ (Figure 4.25).

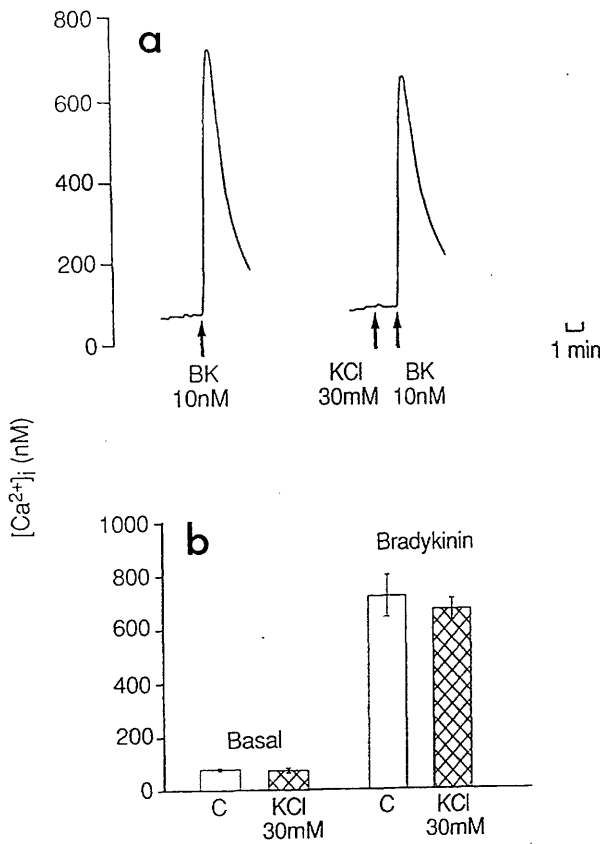


Figure 4.24 Individual traces (a) and a histogram (b) illustrating the effects of pre-treatment with potassium chloride (KCl; 30mM, 1min) on basal levels of $[Ca^{2+}]_i$ and the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by bradykinin (BK; 10nM) in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium. In the histogram, basal and bradykinin-induced elevations of $[Ca^{2+}]_i$ in control cells (open columns) and in potassium chloride-treated cells (cross-hatched columns) are the mean of 5-11 observations and vertical bars indicate the s.e. mean.

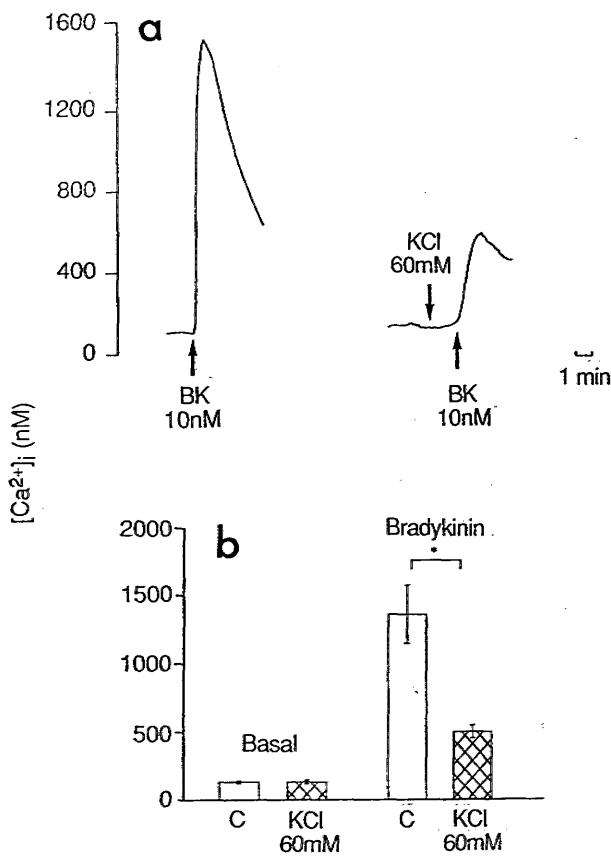


Figure 4.25 Individual traces (a) and a histogram (b) illustrating the effects of pre-treatment with potassium chloride (KCl; 60mM, 1min) on basal levels of $[Ca^{2+}]_i$ and the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by bradykinin (BK; 10nM) in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium. In the histogram, basal and bradykinin-induced elevations of $[Ca^{2+}]_i$ in control cells (open columns) and potassium chloride-treated cells (cross-hatched columns) are the mean of 4-8 observations and vertical bars indicate the s.e. mean. * $p < 0.05$ indicates a significant difference between groups joined by a bracket.

Addition of KCl (30mM) during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by bradykinin (10nM), thrombin ($1U\ ml^{-1}$) and ATP ($30\mu M$) resulted in a transient fall in $[Ca^{2+}]_i$; $[Ca^{2+}]_i$ returned to pre-KCl levels after approximately 90s (Figure 4.26). It is clear from this study, that the effects of KCl on calcium mobilisation in BAEC are complex.

4.4 Protein Kinase C

4.4.1 Effects of protein kinase C stimulation on $[Ca^{2+}]_i$ in BAEC

The tumour-promoting phorbol esters exert their actions via the activation of protein kinase C (Castagna et al., 1982). Several studies have demonstrated the ability of phorbol esters to inhibit endothelium-dependent relaxation (Weinheimer et al., 1986; Lewis & Henderson, 1987; Cherry & Gillis, 1988). The inability of the phorbol esters to inhibit sodium nitroprusside-induced relaxations (Lewis & Henderson, 1987) suggests that these compounds inhibit the production, but not the action of endothelial-derived relaxing factor (EDRF). Furthermore, the finding that endothelium-dependent relaxation induced by the calcium ionophore, A23187, is unaffected by phorbol esters (Weinheimer et al., 1986; Lewis & Henderson, 1987; Cherry & Gillis, 1988) strongly suggests they inhibit EDRF production by blocking receptor-mediated calcium mobilisation. In this study, the calcium sensitive fluorescent probe, fura-2, was used to assess directly

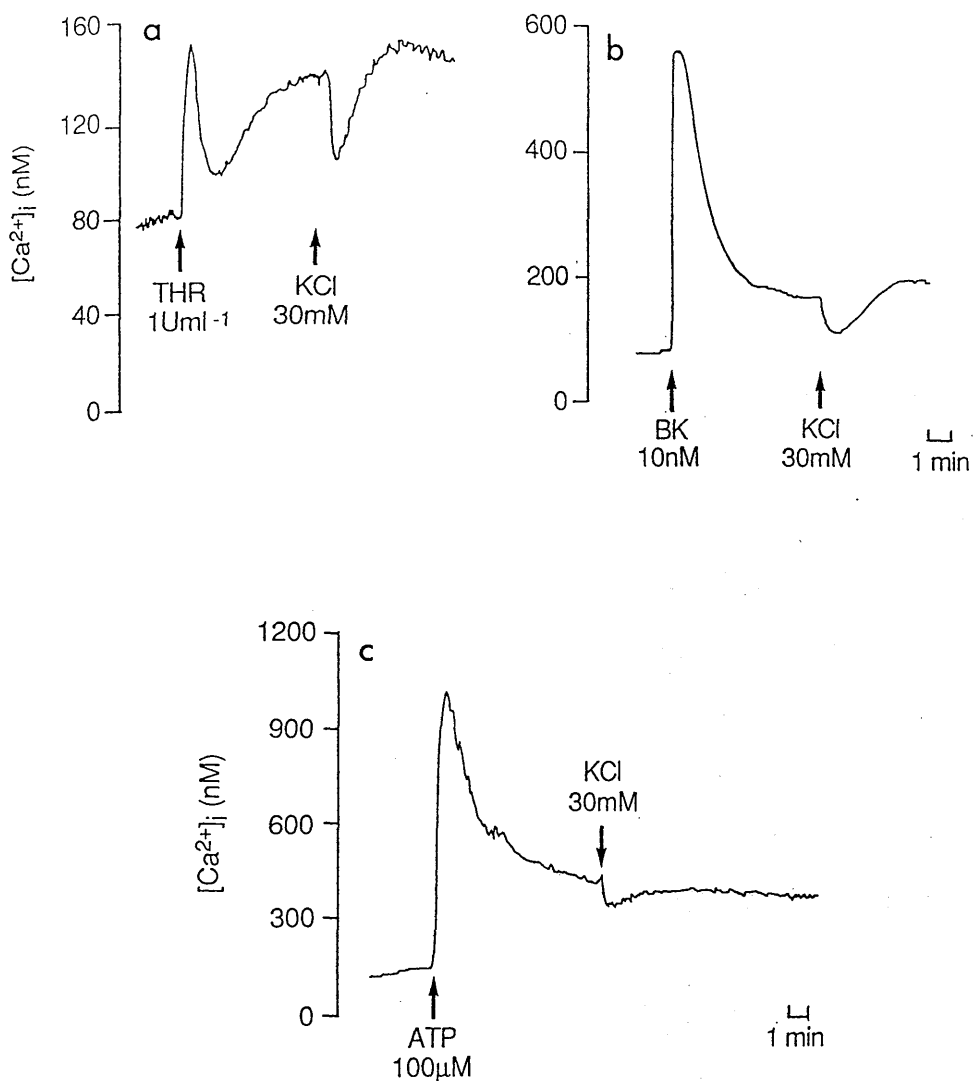


Figure 4.26 Individual traces illustrating the effects of addition of potassium chloride (KCl; 30mM) during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (a; THR; 1U ml⁻¹), bradykinin (b; BK; 10nM) and ATP (c; 100μM) in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium. Each experiment was undertaken on a single occasion.

whether or not phorbol esters could inhibit agonist-induced mobilisation of $[Ca^{2+}]_i$ in bovine aortic endothelial cells.

4.4.2 Effects of 4 β -phorbol 12-myristate 13-acetate on thrombin-induced elevations of $[Ca^{2+}]_i$ in BAEC

In the presence of 1.8mM extracellular calcium, the basal level of $[Ca^{2+}]_i$ in BAEC, in this set of experiments, was 88 ± 5 nM (n=37). Pre-treatment with 4 β -phorbol 12-myristate 13-acetate (PMA; 100nM, 5min), an activator of protein kinase C, had no effect on basal $[Ca^{2+}]_i$ (Figure 4.27; Table 4.1). It did, however, significantly reduce the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin ($1U\ ml^{-1}$; Figure 4.27; Table 4.1). Furthermore, addition of PMA (100nM) during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin ($1U\ ml^{-1}$) induced a fall in $[Ca^{2+}]_i$ of 89 ± 26 nM (n=4) (Figure 4.28).

4.4.3 Effects of 4 β -phorbol 12-myristate 13-acetate on bradykinin-induced elevations of $[Ca^{2+}]_i$ in BAEC

In the presence of 1.8mM extracellular calcium, pre-treatment of BAEC with PMA (100nM, 5min) inhibited the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by bradykinin (BK; 1nM), but not that induced by higher concentrations of bradykinin (3nM and 10nM) (Figures 4.29 and 4.30).

Addition of PMA (100nM) during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by bradykinin (1nM and 10nM)

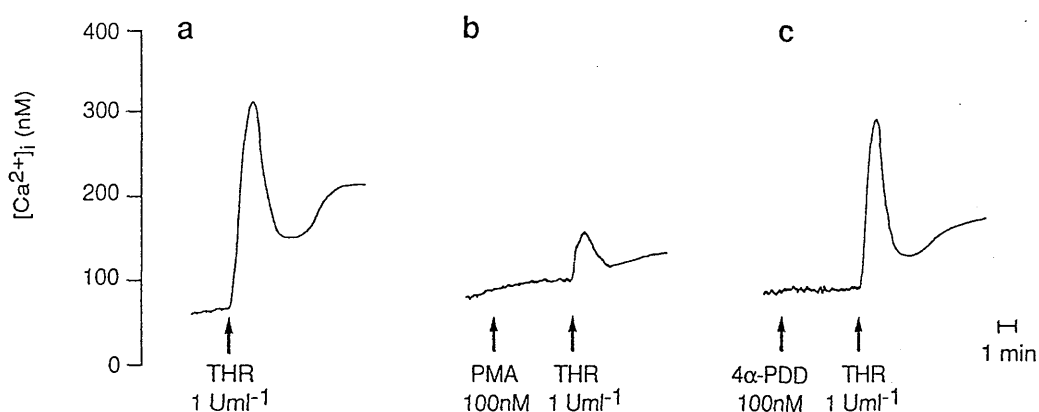


Figure 4.27 Individual traces illustrating the effects of pre-treatment with 4β -phorbol 12-myristate 13-acetate (b; PMA; 100 nM , 5 min) and 4α -phorbol 12,13 didecanoate (c; 4α -PDD; 100 nM , 5 min) on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (a; THR; 1 U ml^{-1}) in bovine aortic endothelial cells, in the presence of 1.8 mM extracellular calcium. Traces (a), (b) and (c) are representative of 20, 10 and 6 experiments, respectively.

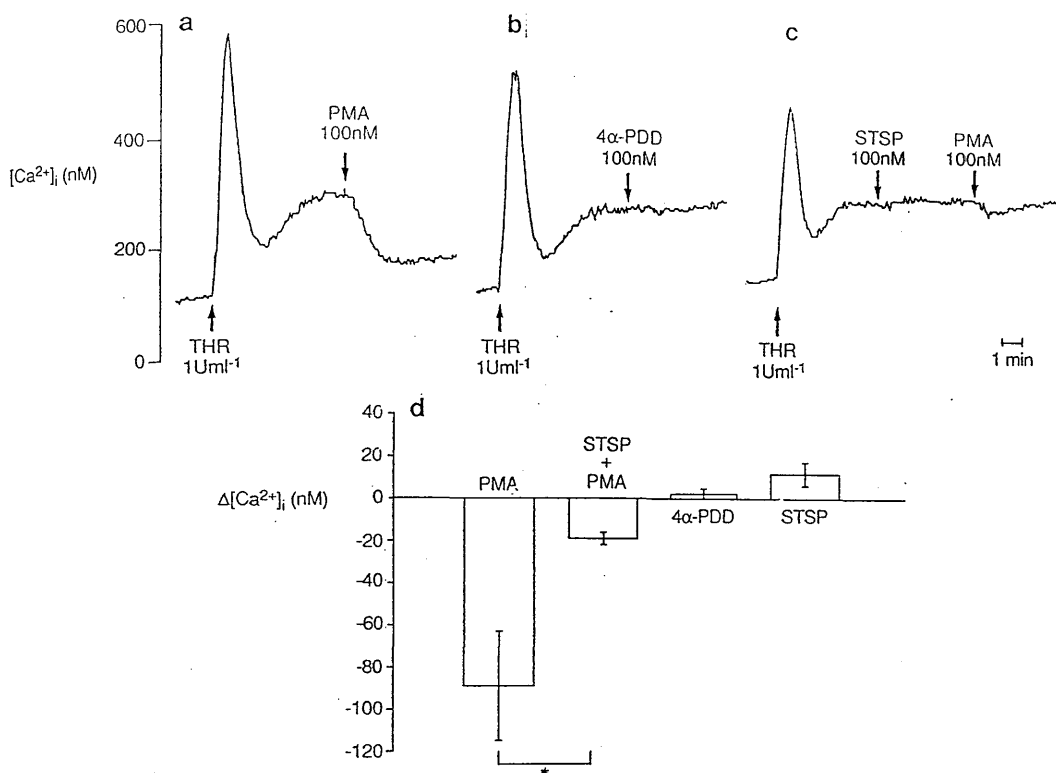


Figure 4.28 Individual traces (a,b,c) and a histogram (d) illustrating the effects of pre-treatment with 4β-phorbol 12-myristate 13-acetate (PMA; 100nM) and 4α-phorbol 12,13 didecanoate (4α-PDD; 100nM) and staurosporine on the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (THR; 1U ml⁻¹) in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium. In the histogram, a positive value illustrates a rise in $[Ca^{2+}]_i$ and a negative value represents a fall in $[Ca^{2+}]_i$. The results shown are the mean of 4-5 observations, vertical bars indicating s.e. mean. * $p < 0.05$ denotes a significant difference between groups joined by a bracket.

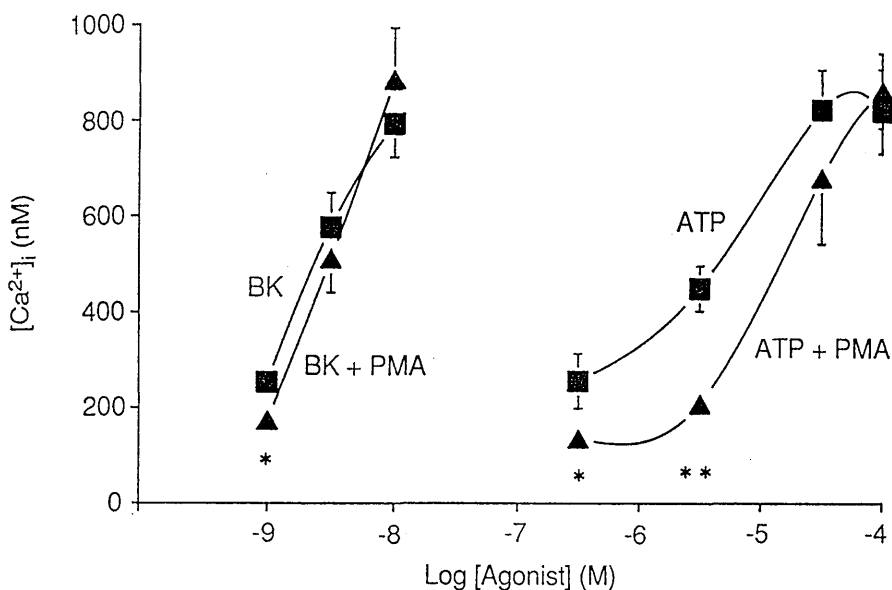


Figure 4.29 Concentration-effect curves showing the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by bradykinin (BK) and adenosine triphosphate (ATP) in bovine aortic endothelial cells in the absence (squares) and presence (triangles) of 4 β -phorbol 12-myristate 13-acetate (PMA; 100nM, 5min). All experiments were undertaken in the presence of 1.8mM extracellular calcium. Individual points represent the mean of 4-10 observations and vertical bars represent the s.e. mean. Where the error bars are not seen, they are encompassed within the symbols. * $p < 0.05$, ** $p < 0.01$, indicates a significant difference from the response obtained in the absence of PMA.

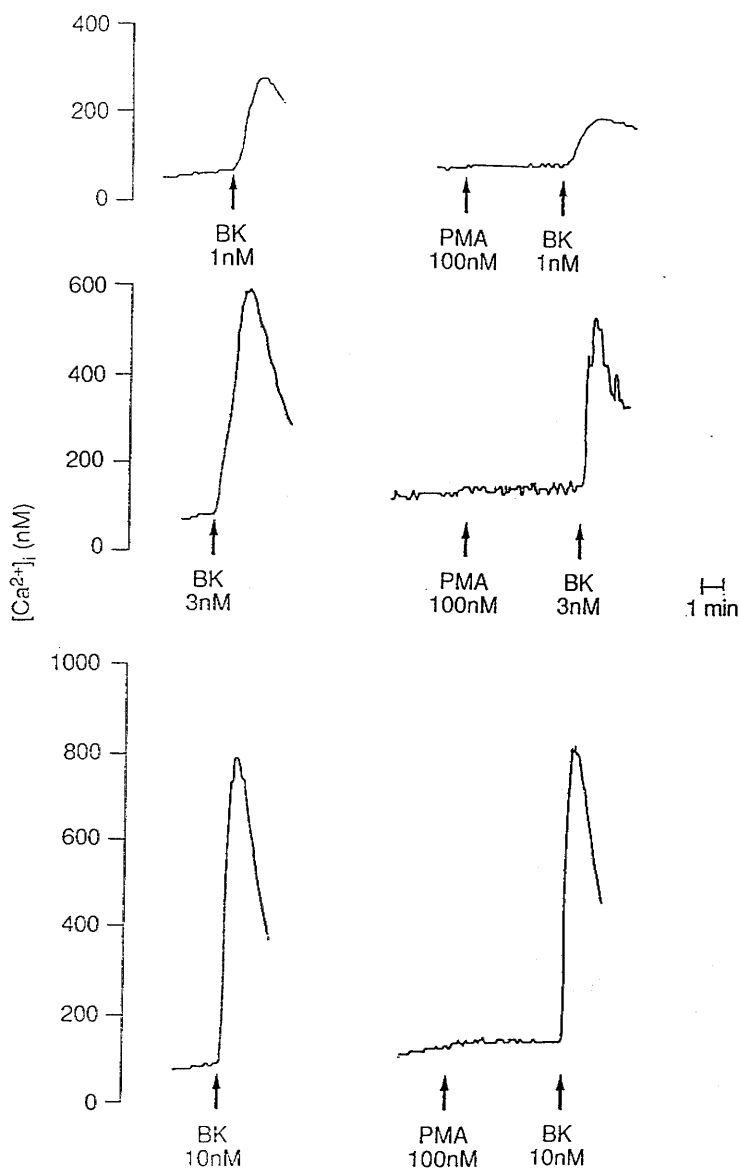


Figure 4.30 Individual traces illustrating the effects of pre-treatment with 4 β -phorbol 12-myristate 13-acetate (PMA; 100nM, 5min) on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by bradykinin (BK; 1nM, 3nM, 10nM) in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium.

Table 4.1

<u>Stimulus</u>	<u>Pre-treatment</u>	$[Ca^{2+}]_i$	<u>n</u>
None (Control)	None	88±5nM	37
None	PMA(100nM)	97±9nM	10
None	STSP(100nM)	102±7nM	13
None	PMA(100nM)		
	+STSP(100nM)	122±4nM *	7
THR(1U ml ⁻¹)(Control)	None	333±29nM	14
THR(1U ml ⁻¹)	PMA(100nM)	191±20nM	10
THR(1U ml ⁻¹)	STSP(100nM)	274±34nM	6
THR(1U ml ⁻¹)	PMA(100nM)		
	+STSP(100nM)	274±24nM	9

Bovine aortic endothelial cells were pre-treated with either 4β-phorbol 12-myristate 13-acetate (PMA; 100nM, 5min), staurosporine (STSP; 100nM, 5min) or with a combination of STSP (100nM, 10min) and PMA (100nM, 5min). The effects of these pre-treatments on both basal $[Ca^{2+}]_i$ and the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (THR; 1U ml⁻¹) are shown. Values are the mean ± s.e.mean. *p<0.05; ***p<0.01 denotes a significant difference from the control or a difference between groups joined by a bracket.

induced falls in $[Ca^{2+}]_i$ of $37 \pm 4 nM$ ($n=6$) and $35 \pm 14 nM$ ($n=4$), respectively (Figure 4.31).

4.4.4 Effects of 4 β -phorbol 12-myristate 13-acetate on ATP-induced elevations of $[Ca^{2+}]_i$ in BAEC

In the presence of 1.8mM extracellular calcium, pre-treatment of BAEC with PMA (100nM, 5min) inhibited the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by ATP (0.3 μ M and 3 μ M), but not those induced by higher concentrations of ATP (30 μ M and 100 μ M) (Figures 4.29 and 4.32).

Addition of PMA (100nM) during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by ATP (0.3 μ M and 30 μ M) induced falls in $[Ca^{2+}]_i$ of $71 \pm 14 nM$ ($n=4$) and $161 \pm 29 nM$ ($n=4$), respectively (Figure 4.33).

4.4.5 Effects of staurosporine on $[Ca^{2+}]_i$ in BAEC

Staurosporine, an inhibitor of protein kinase C (Tamaoki et al., 1986) was used to determine whether PMA exerts its actions via protein kinase C and whether agonist-induced protein kinase C activation may modulate $[Ca^{2+}]_i$ in BAEC.

In the presence of 1.8mM extracellular calcium, treatment with staurosporine (100nM, 5min) had no effect on basal levels of $[Ca^{2+}]_i$ or on the magnitude of the thrombin-induced initial transient elevation of $[Ca^{2+}]_i$ (Table 4.1). Staurosporine (100nM, 10min) did, however, inhibit the ability of PMA (100nM, 5min) to attenuate the thrombin-induced transient elevation of $[Ca^{2+}]_i$ (Table 4.1).

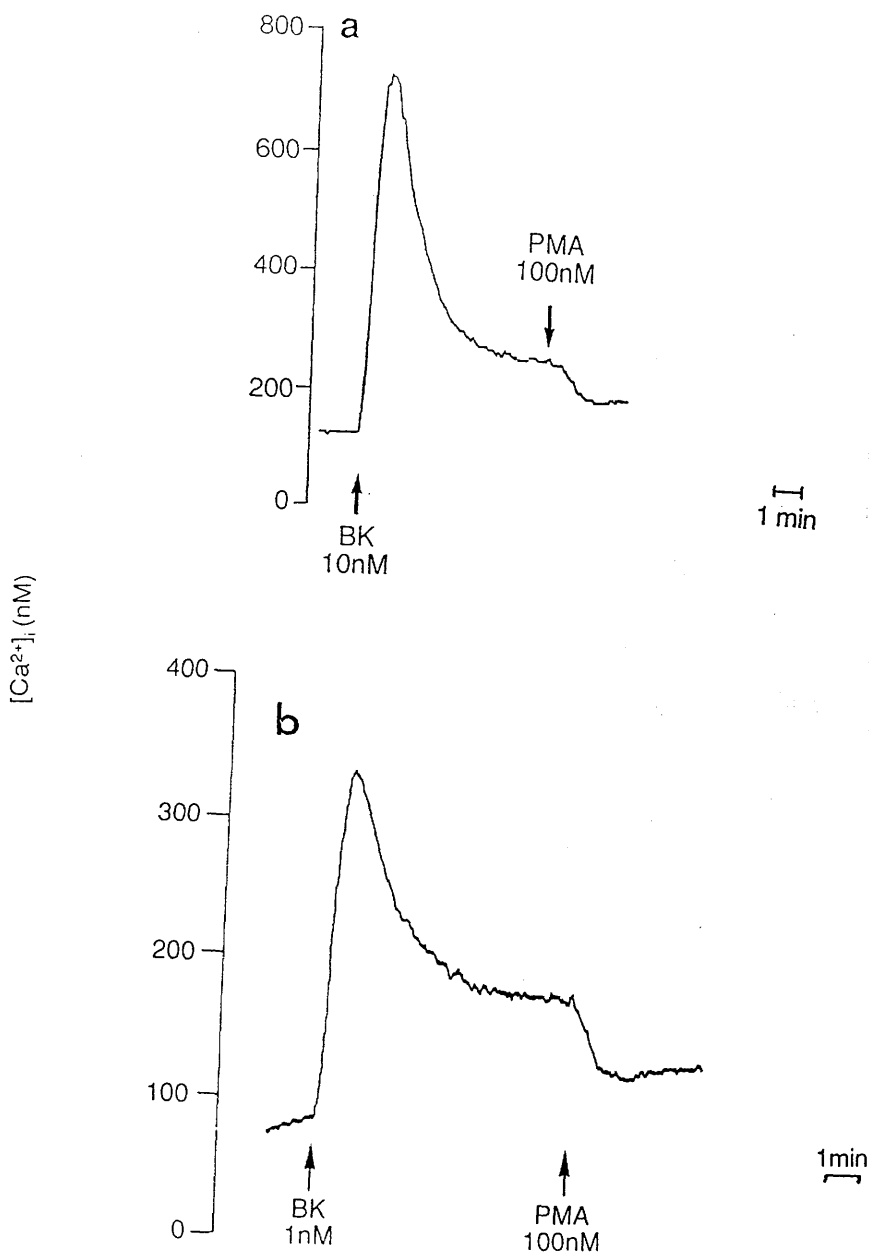


Figure 4.31 Individual traces illustrating the effects of 4 β -phorbol 12-myristate 13-acetate (PMA; 100nM) on the plateau phase of the increase in $[Ca^{2+}]_i$ induced by bradykinin (BK; 10nM, 1nM) in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium. Traces (a) and (b) are typical examples of the action of PMA and were obtained with 6 and 4 batches of cells, respectively.

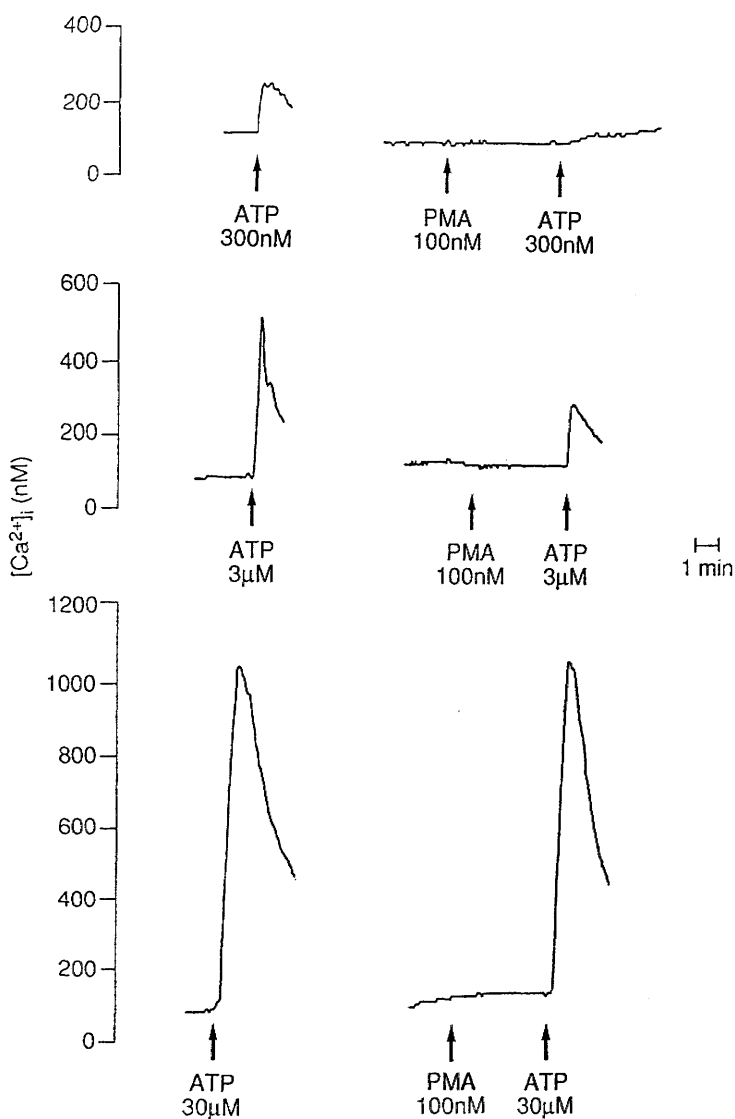


Figure 4.32 Individual traces illustrating the effects of pre-treatment with 4 β -phorbol 12-myristate 13-acetate (PMA; 100nM, 5min) on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by ATP (0.3 μ M, 3 μ M and 30 μ M) in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium.

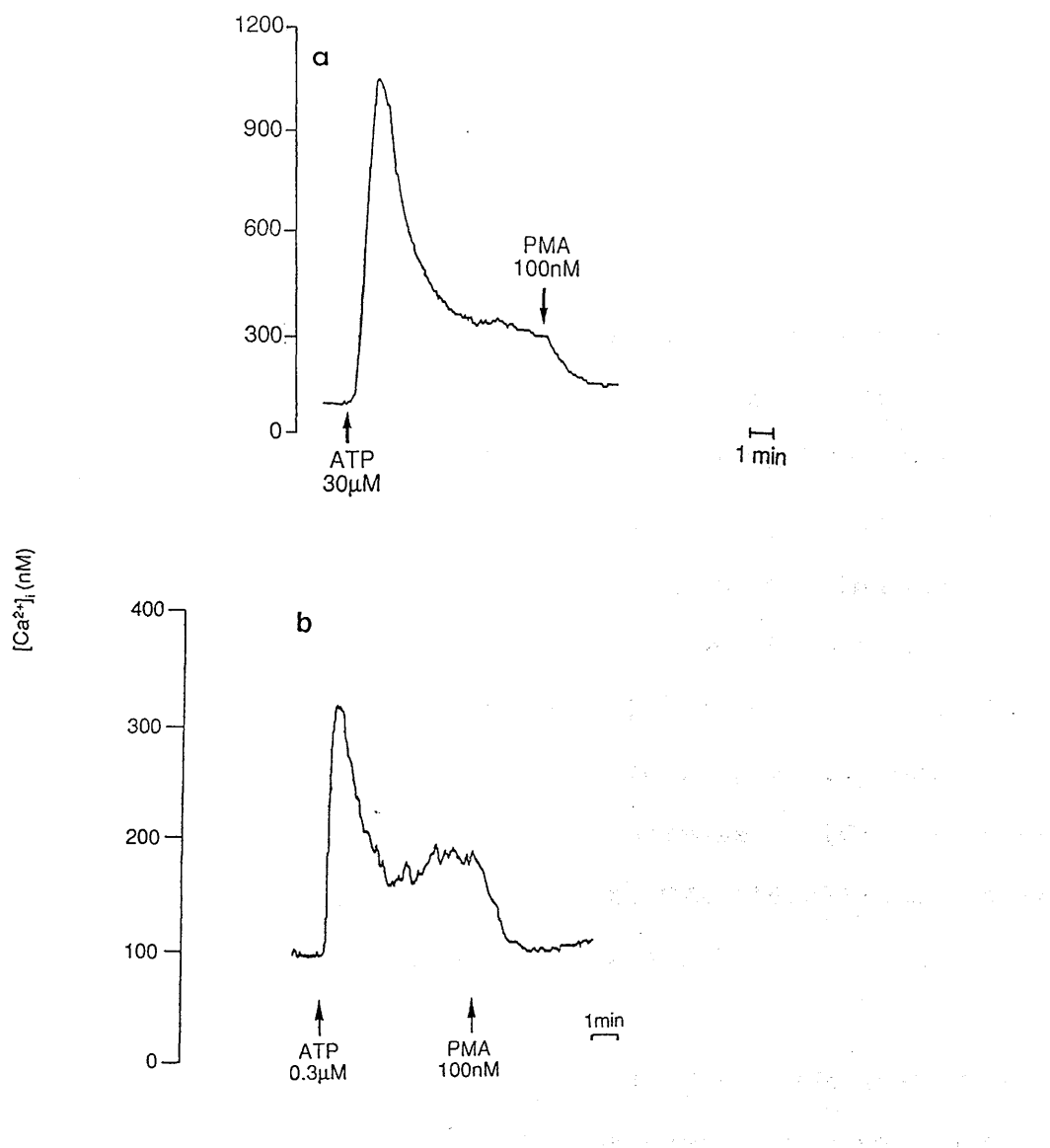


Figure 4.33 Individual traces illustrating the effects of 4 β -phorbol 12-myristate 13-acetate (PMA; 100nM) on the plateau phase of the increase in $[Ca^{2+}]_i$ induced by ATP (30 μM and 0.3 μM) in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium. Traces (a) and (b) are typical examples of the actions of PMA obtained with 4 batches of cells at each concentration of ATP.

In a second series of experiments, staurosporine (100nM, 5min) had no effect on basal $[Ca^{2+}]_i$ or on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by bradykinin (1nM) (Figure 4.34).

Staurosporine (100nM) added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin ($1U\ ml^{-1}$) and bradykinin (10nM) resulted in increases in $[Ca^{2+}]_i$ of $10 \pm 3nM$ ($n=13$) and $43 \pm 12nM$ ($n=4$), respectively (Figure 4.35). In contrast, staurosporine (100nM), induced a fall in $[Ca^{2+}]_i$ of $76 \pm 14nM$ ($n=4$) when added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by ATP ($30\mu M$) (Figure 4.35). Furthermore, staurosporine, when added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin ($1U\ ml^{-1}$), reduced the PMA (100nM)-induced fall in $[Ca^{2+}]_i$ to $19 \pm 3nM$ ($n=4$; Figure 4.28).

From these findings, it is likely that PMA mediates its actions through the activation of protein kinase C and that agonist-induced activation of protein kinase C may regulate $[Ca^{2+}]_i$ in BAEC.

4.4.6 Effects of 4 α -phorbol 12, 13-didecanoate on thrombin-induced elevations of $[Ca^{2+}]_i$ in BAEC

4 α -phorbol 12, 13-didecanoate (4 α -PDD) is a phorbol ester which does not activate protein kinase C (Castagna et al., 1982). Consequently, it can be used to determine if the actions of PMA are mediated through activation of protein kinase C.

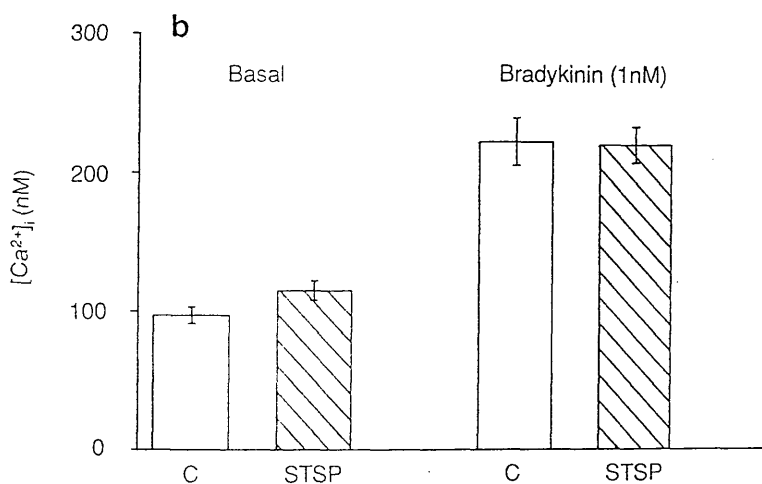
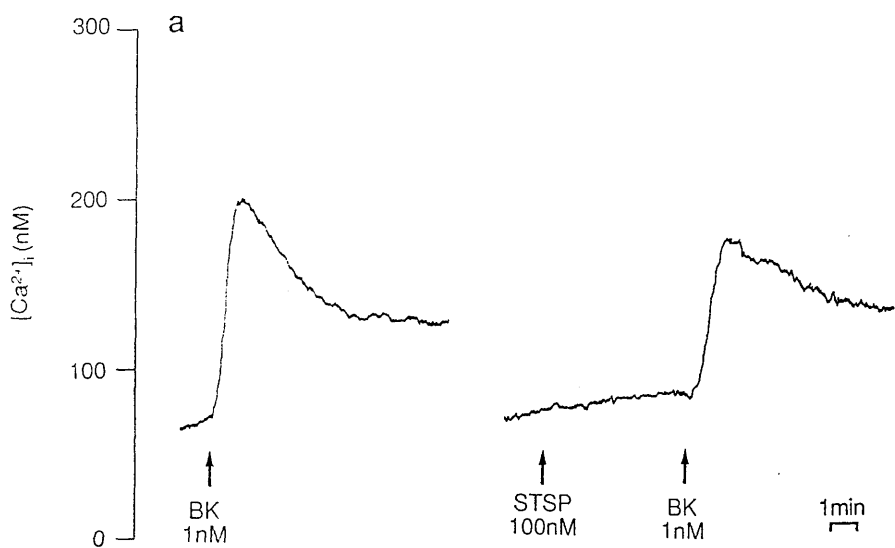


Figure 4.34 Individual traces (a) and a histogram (b) showing the effects of pre-treatment with staurosporine (STSP; 100nM, 5 min) on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by bradykinin in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium. In the histogram, basal levels and bradykinin (BK; 1nM)-induced elevations of $[Ca^{2+}]_i$ in control cells (open column) and staurosporine-treated cells (hatched column) are the mean of 4-8 observations and vertical bars indicate the s.e. mean.

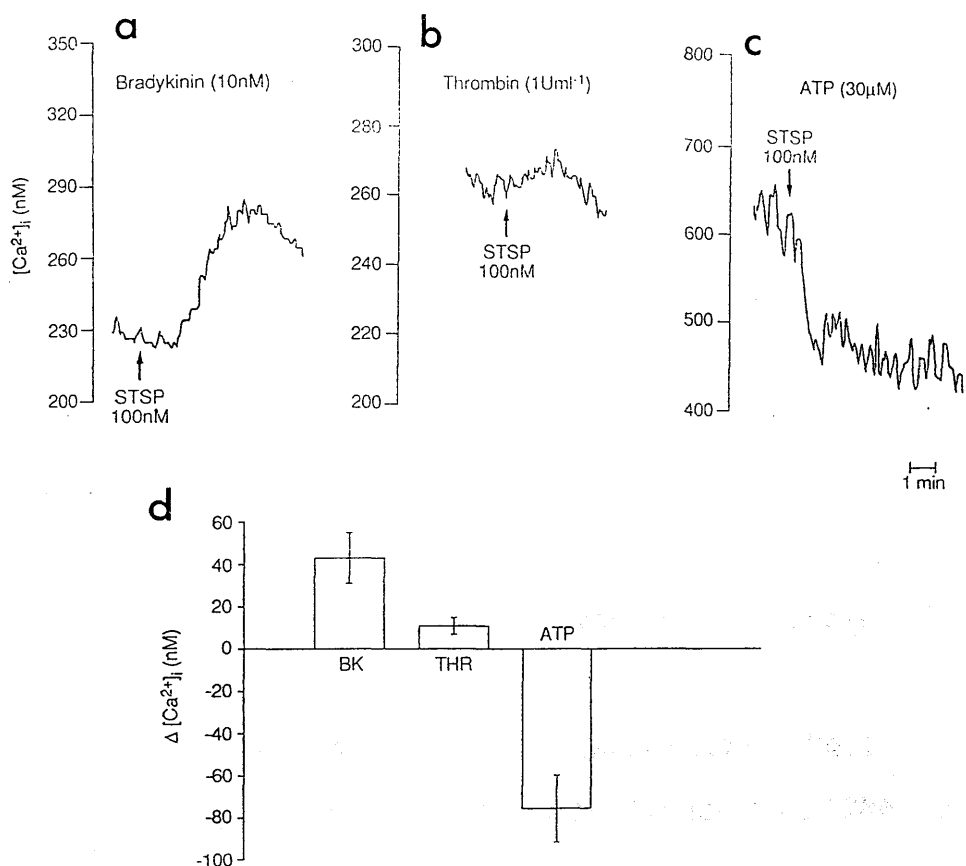


Figure 4.35 Individual traces (a,b and c) and a histogram (d) illustrating the changes in $[Ca^{2+}]_i$ observed when staurosporine (STSP; 100nM) was added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by bradykinin (10nM), thrombin (1U ml⁻¹) and ATP (30μM) in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium. In the histogram, results are given as the mean of 4-13 observations and vertical bars indicate the s.e. mean. A positive value indicates a rise in $[Ca^{2+}]_i$ while a negative value indicates a fall in $[Ca^{2+}]_i$.

In the presence of 1.8mM extracellular calcium, treatment with 4 α -PDD (100nM, 5min) had no effect on basal levels of $[Ca^{2+}]_i$ or on the magnitude of the actual transient elevation of $[Ca^{2+}]_i$ induced by thrombin (1U ml⁻¹) (Figures 4.27 and 4.36).

Addition of 4 α -PDD (100nM) during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (1U ml⁻¹) had no effect on $[Ca^{2+}]_i$ (Figure 4.28).

From these findings, it appears that PMA exerts its action on calcium mobilisation via activation of protein kinase C.

4.4.7 Effects of 4 α -phorbol 12, 13-didecanoate (4 α -PDD) and 4 β -phorbol 12-myristate 13-acetate (PMA) on the thrombin-induced manganese quench of fura-2

Agonist-induced elevations of $[Ca^{2+}]_i$ result from both mobilisation of intracellular calcium pools and from calcium influx across the cell membrane. The ability of receptor-mediated agonists to promote entry of divalent cations has been demonstrated in human umbilical vein endothelial cells by Hallam et al. (1988b). The procedure used to demonstrate entry of divalent cations depends on the ability of endothelial cells to permit entry of manganese (Mn²⁺) through the same channels which allow the entry of calcium. Entry of Mn²⁺ results in the quenching of intracellular fura-2. Although fura-2 responds to changes in calcium at most excitation wavelengths, no change in fluorescence is observed at an excitation

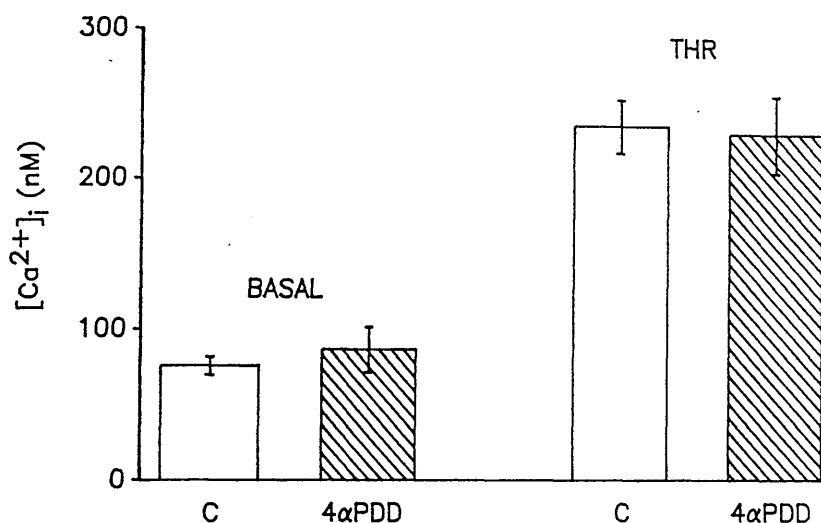


Figure 4.36 Effects of pre-treatment with 4 α -phorbol 12,13 didecanoate (4 α -PDD; 100nM, 5min) on basal levels of $[Ca^{2+}]_i$ and on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (THR; 1U ml⁻¹) in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium. In the histogram, basal levels of $[Ca^{2+}]_i$ and the magnitude of the initial transient elevations of $[Ca^{2+}]_i$ induced by thrombin in control cells (open columns) and in 4 α -PDD-treated cells (hatched columns) are given as the mean of 6-12 observations.

wavelength of 360nm, i.e., the isobestic wavelength. Therefore, the ability of Mn^{2+} to quench fura-2 fluorescence, following excitation at 360nm, provides a measure of divalent cation entry and, by implication, calcium entry. An attempt was made to use this technique to determine whether or not PMA could be shown to inhibit thrombin-induced divalent cation entry into BAEC.

BAEC were incubated in nominally calcium-free HEPES(10mM)-buffered Krebs. Following alternate excitation at 340nm and 360nm, fura-2 fluorescence was collected at 509nm. Addition of the inactive phorbol ester, 4 α -PDD (100nM), had no effect on fura-2 fluorescence (Figure 4.37). Addition of Mn^{2+} (25 μ M) resulted in an increase in the rate of fall of the fluorescence signal produced by excitation at both 340nm and 360nm (Figure 4.37), reflecting Mn^{2+} entry, and quenching of fura-2 fluorescence. At an excitation wavelength of 360nm, the quenching of fura-2 fluorescence was further accelerated by the addition of thrombin (1U ml⁻¹; Figure 4.37). In contrast, at an excitation wavelength of 340nm, a transient increase in fluorescence was obtained upon addition of thrombin, followed by an increased quenching of fluorescence (Figure 4.37).

In a separate series of experiments, the addition of PMA (100nM) had no effect on fura-2 fluorescence (Figure 4.37). Addition of Mn^{2+} (25 μ M) resulted in an increase in the rate of fall of the fluorescence signals induced by excitation

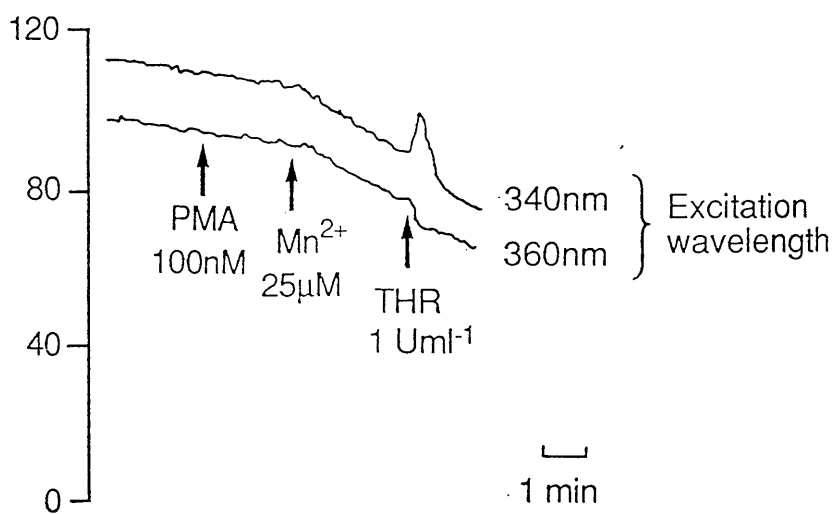
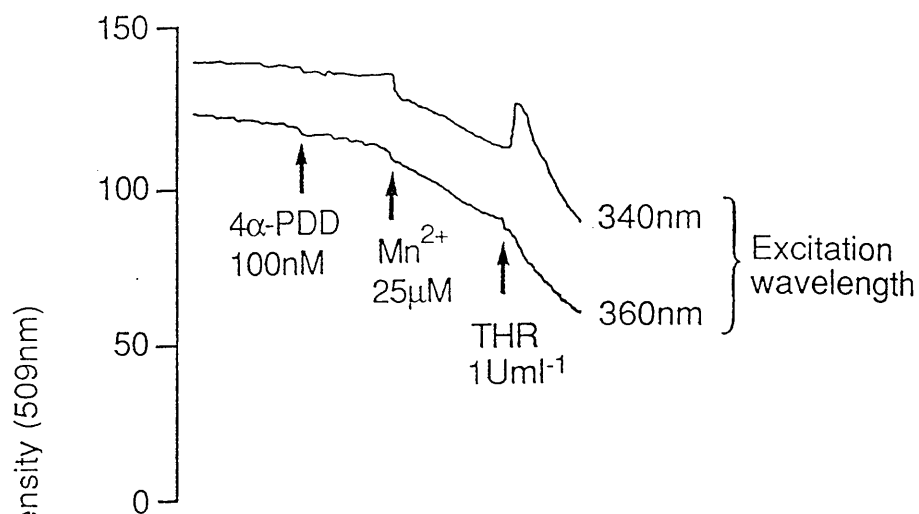


Figure 4.37 Individual traces illustrating the effects of 4 α -phorbol 12,13 - didecanoate (4 α -PDD; 100nM) and 4 β -phorbol 12-myristate 13-acetate (PMA; 100nM) on thrombin (1U ml⁻¹)-stimulated manganese (Mn²⁺) quenching of fura-2 in bovine aortic endothelial cells, in nominally calcium-free buffer. The fura-2-loaded endothelial cells were alternately excited at 340nm and 360nm and the resultant fluorescence emission collected at 509nm. The ability of Mn²⁺ to quench fura-2 fluorescence following excitation at 360nm, the isobestic wavelength for fura-2, was used as a marker for Mn²⁺ entry into the cytosol. Addition of Mn²⁺ (25 μ M; traces a and b) accelerated the rate decay of the fura-2 signal at 360nm, as a result of Mn²⁺ quenching of cytosolic fura-2. Following pre-treatment with 4 α -PDD (100nM, 5min), thrombin (1U ml⁻¹) enhanced the rate of quenching of fura-2 by Mn²⁺. In contrast, pre-treatment with PMA (100nM, 5min) almost completely abolished the ability of thrombin to enhance Mn²⁺ quenching of fura-2.

at 340nm and 360nm (Figure 3.47). At an excitation wavelength of 360nm, the rate of quenching of fura-2 fluorescence by Mn^{2+} was almost unaffected by the addition of thrombin ($1U\ ml^{-1}$; Figure 4.37). At an excitation wavelength of 340nm, a transient increase in fluorescence was obtained upon addition of thrombin (Figure 4.37).

These findings suggest that PMA, but not 4α -PDD, inhibits thrombin-induced calcium influx in BAEC.

4.4.8 Effects of 4β -phorbol 12-myristate 13-acetate on ionomycin-induced elevations of $[Ca^{2+}]_i$ in BAEC

In some cell types (for example, neutrophils), phorbol esters may activate the plasma membrane Ca^{2+} -ATPase (Lagast et al., 1984). Experimentally, this may be demonstrated by the ability of phorbol esters to inhibit calcium mobilisation induced by a calcium ionophore (McCarthy et al., 1989). Therefore, we wished to determine if promotion of calcium efflux could be observed in BAEC.

In the presence of 1.8mM extracellular calcium, PMA (100nM) has no effect on the ionomycin (10nM)-induced sustained elevation of $[Ca^{2+}]_i$ (Figure 4.38). Therefore, it is unlikely that phorbol esters activate the plasma membrane Ca^{2+} -ATPase in BAEC.

4.5 **Cyclic AMP**

4.5.1 Effects of cyclic AMP in the regulation of $[Ca^{2+}]_i$

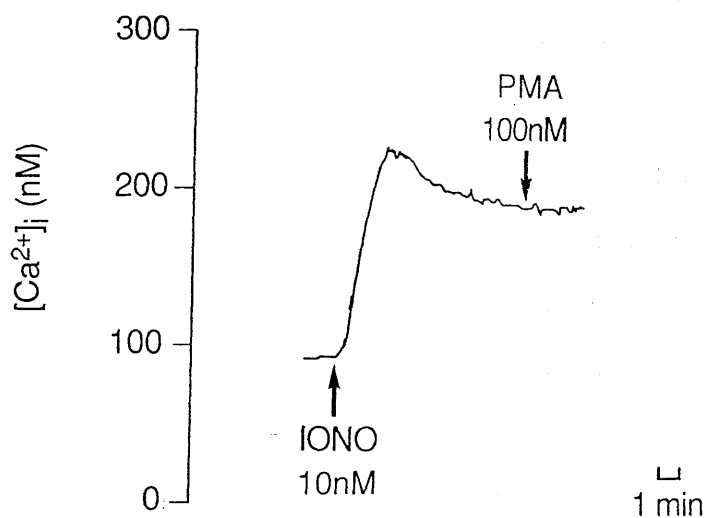


Figure 4.38 Individual trace illustrating the effects of 4 β -phorbol 12-myristate 13-acetate (PMA; 100nM) on the sustained elevation of $[Ca^{2+}]_i$ induced by ionomycin (IONO; 10nM), in the presence of 1.8mM extracellular calcium. This trace is representative of 2 separate experiments.

in BAEC

Cyclic AMP is known to regulate cell function via the modulation of calcium mobilisation in a number of cell types. For example, elevation of cyclic AMP has been shown to inhibit calcium mobilisation in vascular smooth muscle (Meisheri & van Breemen, 1982) and in platelets (MacIntyre et al., 1985b), but to enhance calcium mobilisation in other cell types, such as hepatocytes (Poggioli et al., 1986), T-lymphocytes (Kelley et al., 1990) and cardiac myocytes (Cachelin et al., 1983). Endothelial cyclic AMP levels can be elevated by several agents, including prostacyclin (Dembinska - Kiec et al., 1980; Hopkins & Gorman, 1981), isoprenaline (Buonassi & Venter, 1976; Hopkins & Gorman, 1981) and forskolin (Adams Brotherton et al., 1982). Little is known about how these changes in cyclic AMP levels modulate either endothelial intracellular signalling or endothelial cell function. In this section, the effects of elevating cyclic AMP levels on endothelial calcium mobilisation were investigated.

4.5.2 Effects of forskolin and isoprenaline on thrombin-induced elevations of $[Ca^{2+}]_i$ in BAEC

Forskolin is a diterpene which has been shown to directly activate the catalytic subunit of adenylate cyclase (Seamon et al., 1981). In the presence of 1.8mM extracellular calcium, pre-treatment with forskolin (10 μ M, 5min) had no effect on basal levels of $[Ca^{2+}]_i$ or on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (1U ml⁻¹; Figure 4.39). Addition of forskolin

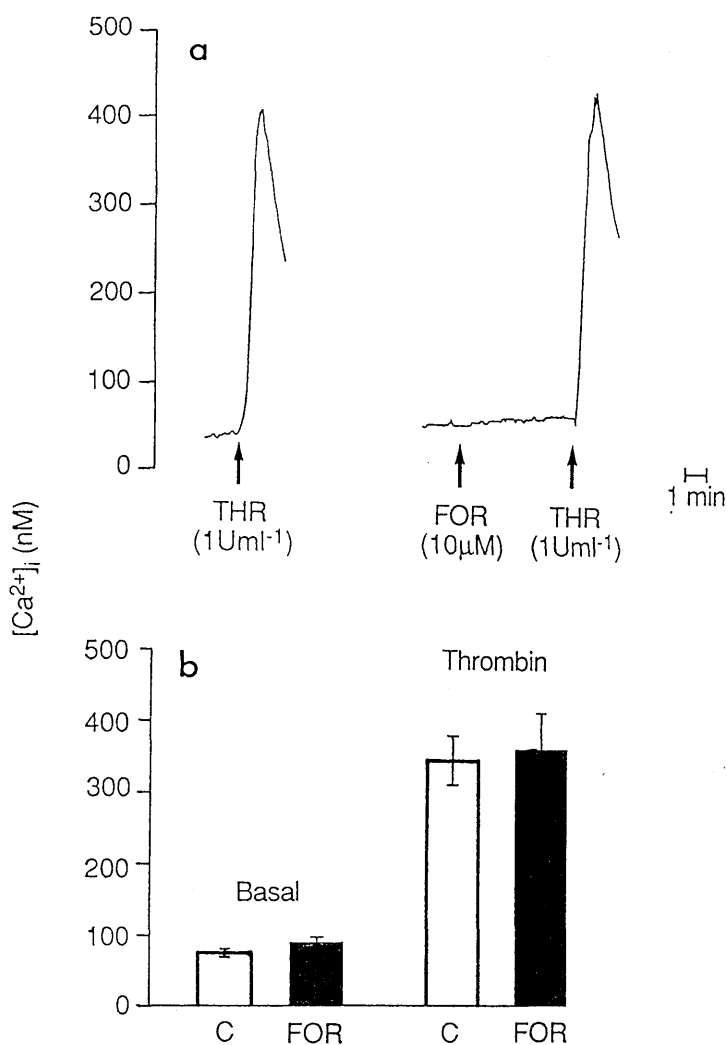


Figure 4.39 Individual traces (a) and a histogram (b) illustrating the effects of pre-treatment with forskolin (FOR; 10 μ M, 5min) on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (THR; 1U ml⁻¹) in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium. In the histogram, basal levels and the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ in control cells (open columns) and forskolin-treated cells (filled columns) are given as the mean of 9-20 observations; the vertical bars indicate the s.e. mean.

(10 μ M) during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (1U ml $^{-1}$) resulted in a biphasic elevation of $[Ca^{2+}]_i$: the maximum increase was 66 \pm 8nM (n=9) and this decayed to 41 \pm 6nM after 5 min (Figure 4.40). Isoprenaline (10 μ M), a β -adrenoceptor agonist, induced a similar biphasic elevation of $[Ca^{2+}]_i$ when added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (1U ml $^{-1}$; Figure 4.40) : the maximum increase was 61 \pm 6nM (n=6) and this decayed to 25 \pm 6nM after 5 min. Following simulation with thrombin (1U ml $^{-1}$) and the addition of nickel (4mM), which blocks calcium influx, forskolin (10 μ M) and isoprenaline (10 μ M) both induced only monophasic elevations of $[Ca^{2+}]_i$ and the plateau phase was abolished (Figure 4.41). As nickel blocks calcium influx, then this demonstrates the ability of forskolin and isoprenaline to enhance calcium mobilisation from intracellular stores.

4.5.3 Effects of staurosporine on forskolin-induced augmentations of thrombin-induced elevations of $[Ca^{2+}]_i$ in BAEC

It has been proposed that staurosporine may inhibit cyclic AMP-dependent protein kinase, in addition to inhibiting protein kinase C and, therefore, its actions are relatively non-selective (Davis et al., 1989). We therefore wished to determine whether staurosporine, at a concentration which inhibits activation of protein kinase C (Section 4.4.5), was able to inhibit the forskolin-induced augmentation of $[Ca^{2+}]_i$ in BAEC.

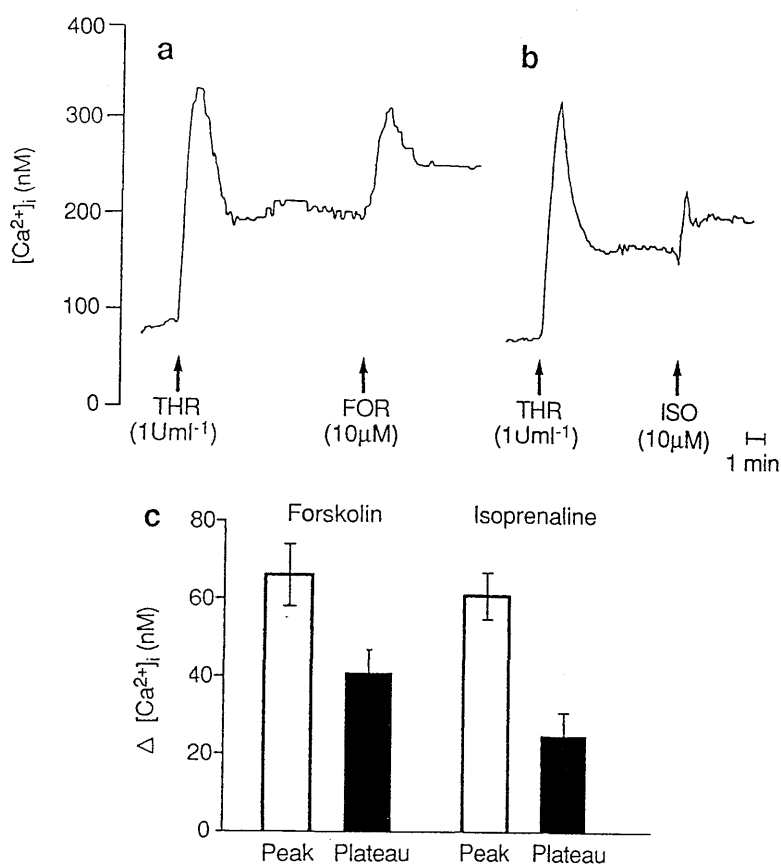


Figure 4.40 Individual traces (a,b) and a histogram (c) illustrating the effects of the addition of forskolin (10μM) and isoprenaline (10μM) during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (THR; 1U ml⁻¹) in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium. In the histogram, the peak elevation of $[Ca^{2+}]_i$ represents the maximum increase in $[Ca^{2+}]_i$ induced by forskolin and isoprenaline, while the plateau elevation of $[Ca^{2+}]_i$ indicates the magnitude of the increase in $[Ca^{2+}]_i$, 5 min after the addition of forskolin and isoprenaline. Results are presented as the mean of 6-9 observations and a vertical bar indicates the s.e. mean.

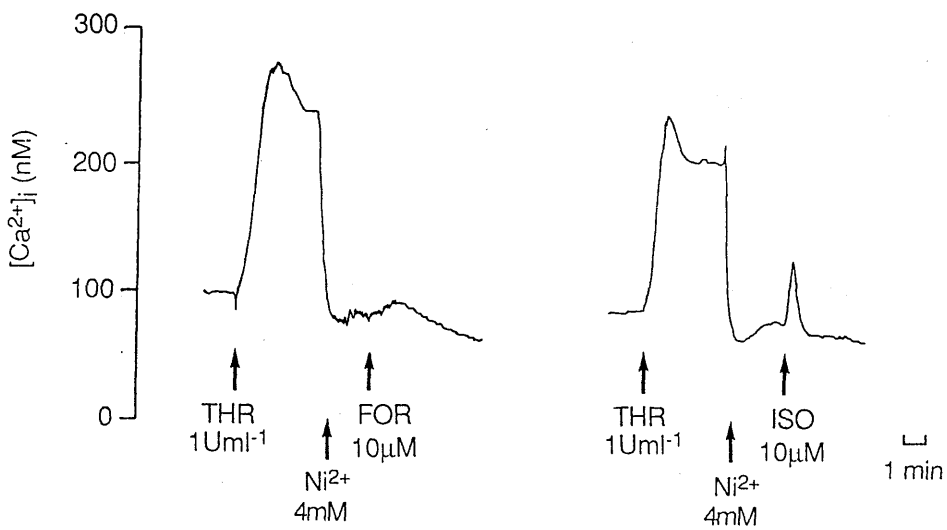


Figure 4.41 Individual traces illustrating the effects of forskolin (FOR; 10 µM) and isoprenaline (ISO; 10 µM) on thrombin (THR; 1 U ml⁻¹)-induced mobilisation of $[Ca^{2+}]_i$ in presence of nickel (Ni²⁺; 4 mM). The traces shown are each typical of 2 separate experiments.

In the presence of 1.8mM extracellular calcium, the addition of forskolin (10 μ M), 700s after addition of thrombin (1U ml⁻¹) induced a biphasic elevation of [Ca²⁺]_i: the maximum increase was 79 \pm 12nM (n=8). Addition of staurosporine (100nM), 400s after thrombin (1U ml⁻¹), induced a small elevation of [Ca²⁺] of 8 \pm 3nM (n=8). Addition of forskolin (10nM), 300s after staurosporine, increased [Ca²⁺]_i by 53 \pm 7nM (n=8). Although apparently slightly smaller than that obtained in cells which did not receive staurosporine, the difference between the forskolin responses was not significant. The small, apparent reduction in the increase in [Ca²⁺]_i, in the presence of staurosporine, may be accounted for by the observation that staurosporine itself induced a small elevation of [Ca²⁺]_i. The total increase in [Ca²⁺]_i induced by staurosporine and forskolin together was 61 \pm 9nM (n=8) which was also not significantly different from that induced by forskolin in cells not receiving staurosporine. Therefore, it appears that the concentration of staurosporine used in this study does not modulate cyclic AMP-mediated responses.

4.5.4 Effects of forskolin and isoprenaline on bradykinin-induced elevations of [Ca²⁺]_i in BAEC

In the presence of 1.8mM extracellular calcium, pre-treatment with forskolin (10 μ M, 5min) had no effect on basal [Ca²⁺]_i or the magnitude of the initial transient elevation of [Ca²⁺]_i induced by bradykinin (1nM and 10nM; Figure 4.42). Pre-treatment with isoprenaline (10 μ M, 5min) also had no effect on basal [Ca²⁺]_i (Figure 4.43) or on the

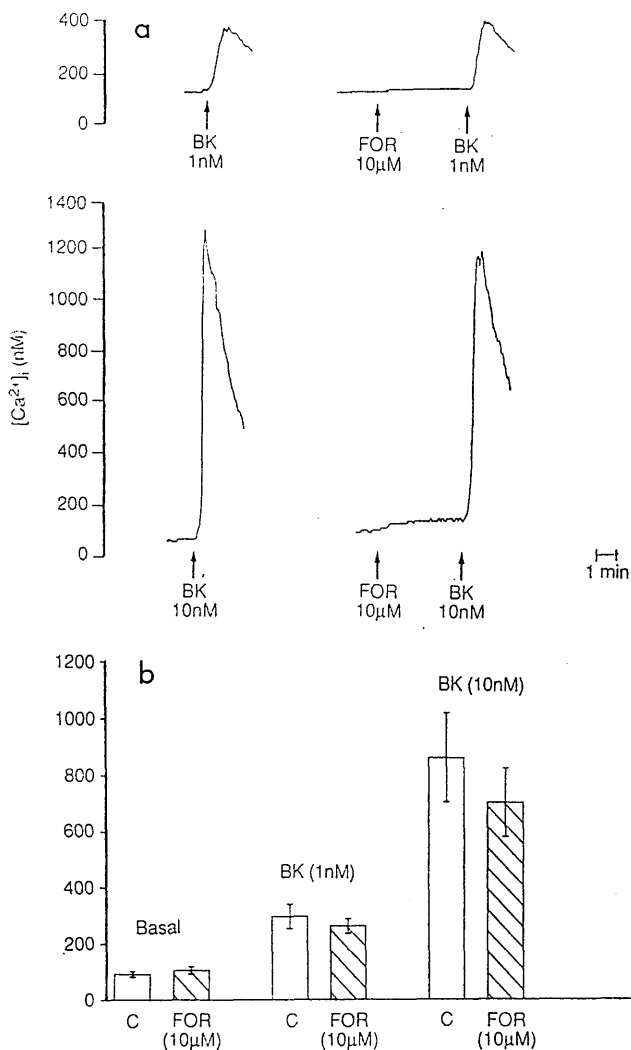


Figure 4.42 Individual traces (a) and a histogram (b) illustrating the effects of pre-treatment with forskolin (FOR; 10μM, 5min) on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by bradykinin (BK; 1nM and 10nM) in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium. In the histogram, basal levels and the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ in control cells (open columns) and forskolin-treated cells (hatched columns) are given as the mean of 4-23 observations; the vertical bars indicate s.e. mean.

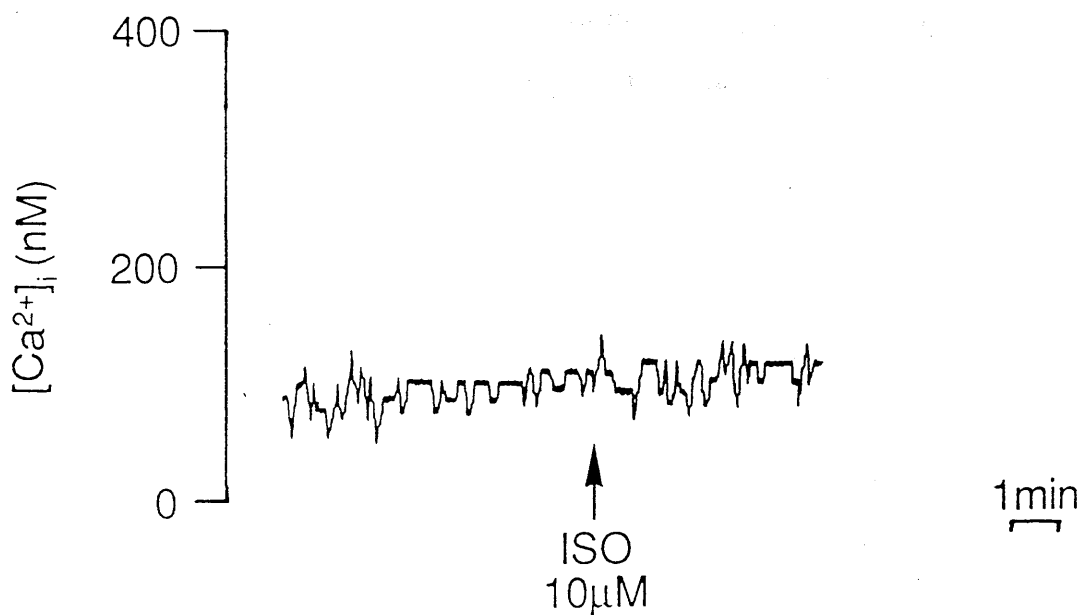


Figure 4.43 Individual trace illustrating the lack of effect of isoprenaline (ISO; 10 μ M) on $[Ca^{2+}]_i$ in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium. The trace shown is representative of 4 separate experiments.

magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by bradykinin (3nM; Figure 4.44).

Addition of forskolin (10 μ M) induced a biphasic elevation of $[Ca^{2+}]_i$ when added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by bradykinin (10nM; Figure 4.45) : the maximum increase was 101 \pm 8nM (n=4) and this decayed to 35 \pm 8nM after 5 min. Isoprenaline (10 μ M) induced a similar biphasic elevation of $[Ca^{2+}]_i$ when added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by bradykinin (10nM; Figure 4.45) : the maximum increase was 130 \pm 18nM (n=4) and this decayed to 16 \pm 8nM after 5 min.

In the presence of nickel (4mM), which blocks calcium influx, the forskolin (10 μ M) - and isoprenaline (10 μ M)-induced elevations of $[Ca^{2+}]_i$ became monophasic, ie, the plateau phase was abolished (Figure 4.46). These actions of nickel suggest that the biphasic response is derived from both intracellular and extracellular calcium pools.

4.5.5 Effects of forskolin and isoprenaline on ATP-induced elevations of $[Ca^{2+}]_i$ in BAEC

In the presence of 1.8mM extracellular calcium, pre-treatment with forskolin (10 μ M, 5min) had no effect on basal $[Ca^{2+}]_i$ or on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by ATP (30 μ M; Figure 4.47).

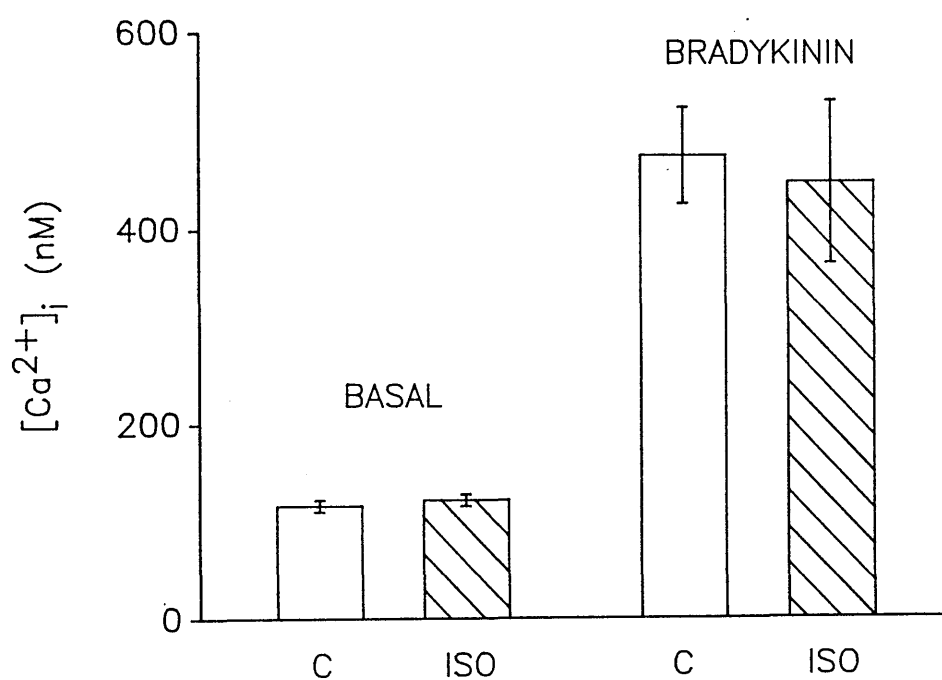


Figure 4.44 Histogram illustrating the effects of pre-treatment with isoprenaline (ISO; $10\mu M$, 5min) on basal $[Ca^{2+}]_i$ and the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by bradykinin (3nM) in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium.

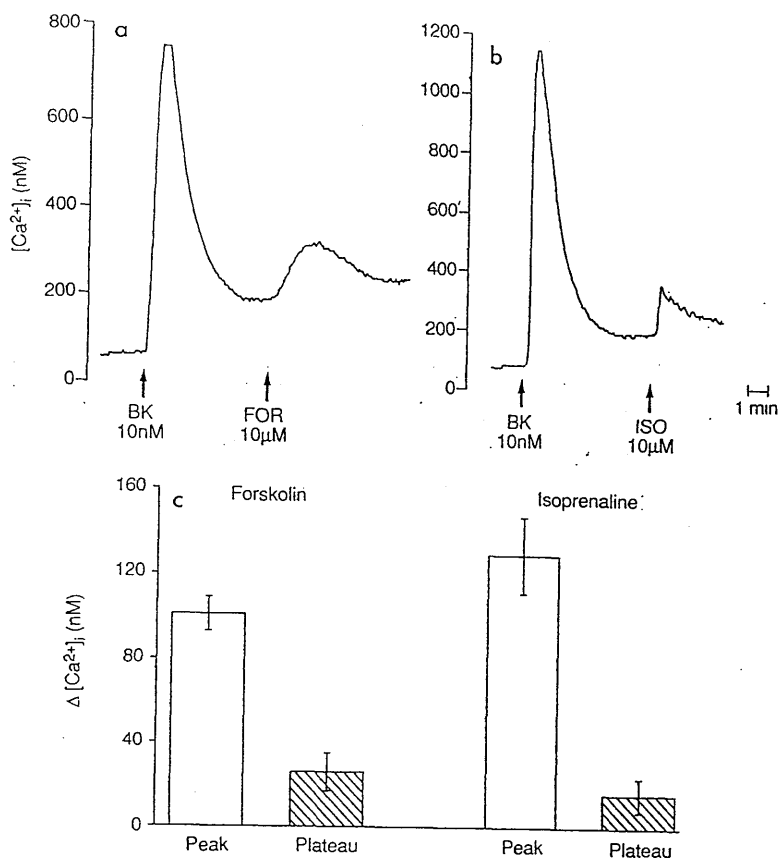


Figure 4.45 Individual traces (a,b) and a histogram (c) illustrating the effects of the addition of forskolin (10μM) and isoprenaline (10μM) during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by bradykinin (BK; 10nM) in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium. In the histogram, the peak elevation of $[Ca^{2+}]_i$ represents the maximum increase in $[Ca^{2+}]_i$ induced by forskolin and isoprenaline, while the plateau elevation of $[Ca^{2+}]_i$ indicates the magnitude of the increase in $[Ca^{2+}]_i$, 5 min after the addition of forskolin and isoprenaline. Results are presented as the mean of 4 observations and a vertical bar indicates the s.e. mean.

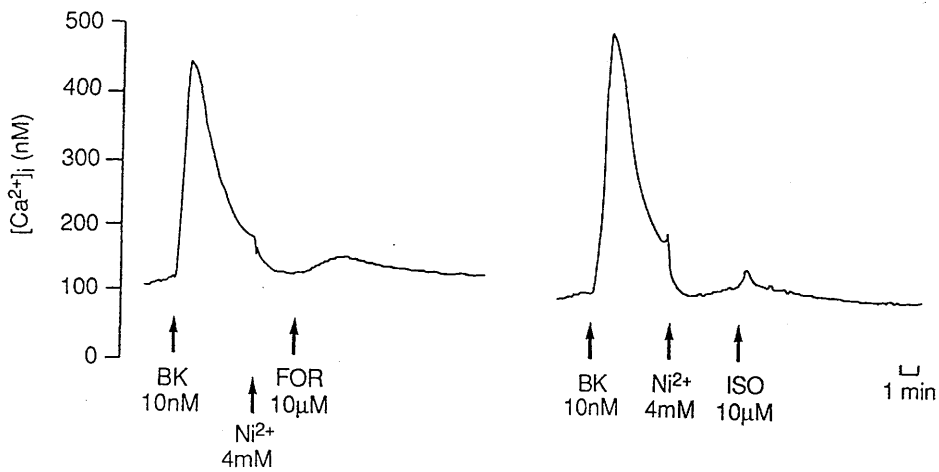


Figure 4.46 Individual traces illustrating the effects of forskolin (FOR; 10µM) and isoprenaline (ISO; 10µM) on bradykinin (BK; 10nM) - induced mobilisation of $[Ca^{2+}]_i$ in the presence of nickel (Ni²⁺; 4mM). The traces shown are each typical of 2 separate experiments.

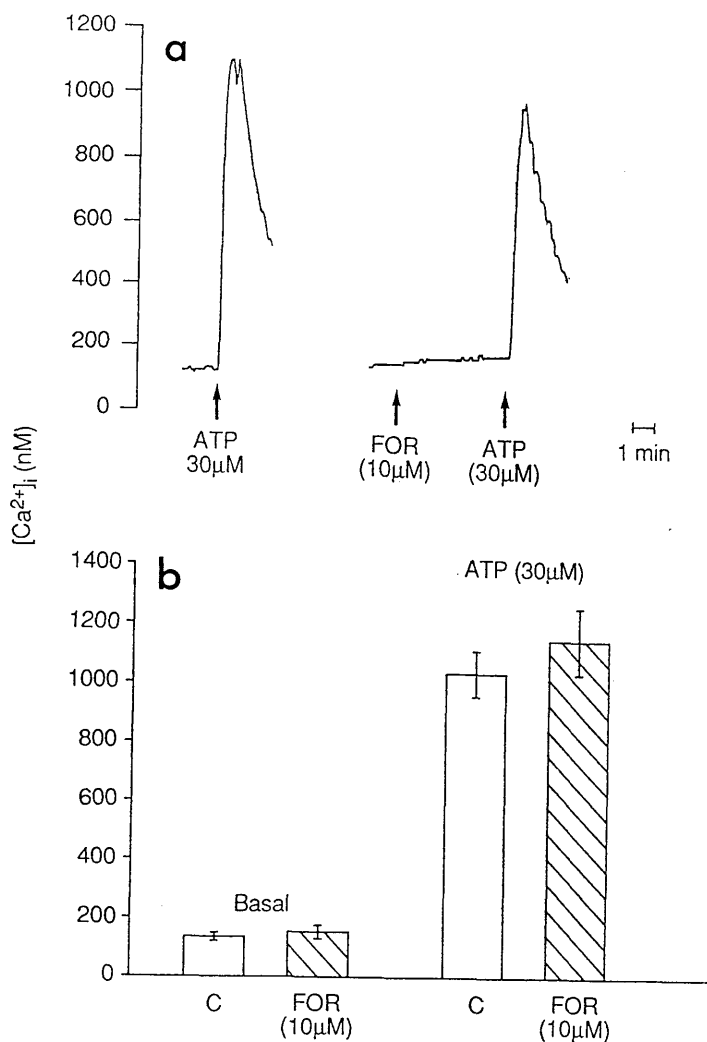


Figure 4.47 Individual traces (a) and a histogram (b) illustrating the effects of pre-treatment with forskolin (10 μ M; 5min) on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by ATP (30 μ M) in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium. In the histogram, basal levels and the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ in control cells (open columns) and forskolin-treated cells (hatched columns) are given as the mean of 4-8 observations; the vertical bars indicate s.e. mean.

Addition of forskolin ($10\mu\text{M}$) during the plateau phase of the increase in $[\text{Ca}^{2+}]_i$ induced by ATP ($3\mu\text{M}$) resulted in a biphasic elevation of $[\text{Ca}^{2+}]_i$ (Figure 4.48) : the maximum increase was $74\pm 13\text{nM}$ ($n=7$) and this decayed to $26\pm 7\text{nM}$ within 5 min. Addition of isoprenaline ($10\mu\text{M}$) during the plateau phase of the increase in $[\text{Ca}^{2+}]_i$ induced by ATP ($3\mu\text{M}$) similarly resulted in a biphasic elevation of $[\text{Ca}^{2+}]_i$ ($n=1$; Figure 4.48).

4.5.6 Effects of forskolin on the ability of PMA to inhibit thrombin-induced calcium mobilisation

As elevation of cyclic AMP has previously been shown to inhibit the actions of protein kinase C in the endothelium (Gudgeon & Martin, 1989) and protein kinase C has been shown to inhibit calcium mobilisation (section 4.4), we wished to determine whether the ability of forskolin to enhance calcium mobilisation derived from the cyclic AMP-mediated inhibition of protein kinase C activity.

However, pre-treatment with forskolin ($10\mu\text{M}$; 5min) did not affect the ability of PMA (100nM) to induce a fall in the plateau phase of the increase in $[\text{Ca}^{2+}]_i$ induced by thrombin (Figure 4.49) : the falls induced by PMA in untreated cells and forskolin-treated cells were $88\pm 8\text{nM}$ ($n=5$) and 114 ± 18 ($n=5$), respectively.

4.6 **Cyclic GMP**

4.6.1 Effects of cyclic GMP in the regulation of $[\text{Ca}^{2+}]_i$ in BAEC

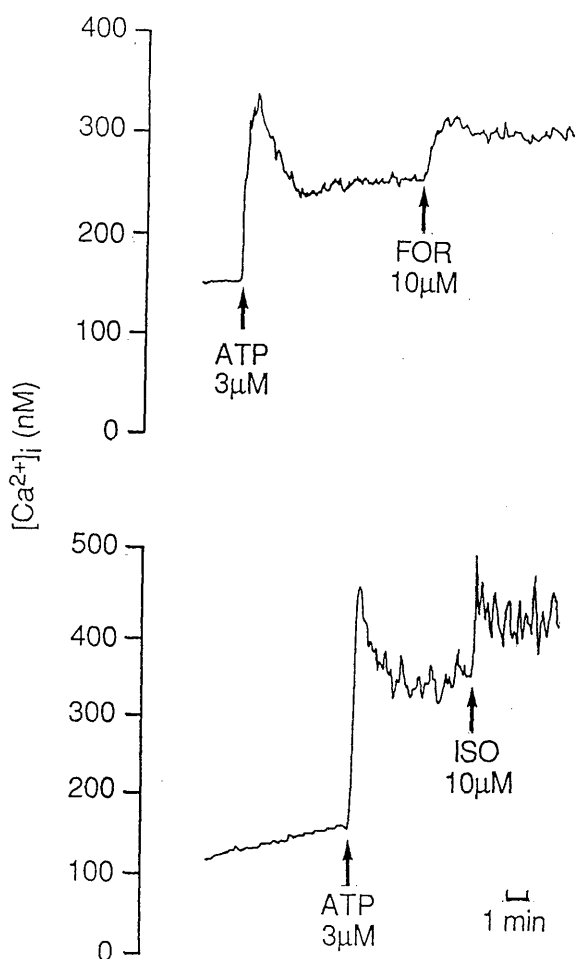


Figure 4.48 Individual traces illustrating the effects of forskolin (FOR; 10 μ M) and isoprenaline (ISO; 10 μ M) on $[Ca^{2+}]_i$ when added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by ATP (3 μ M) in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium. The forskolin experiment is typical of 7 separate experiments while the effects of isoprenaline were only examined once.

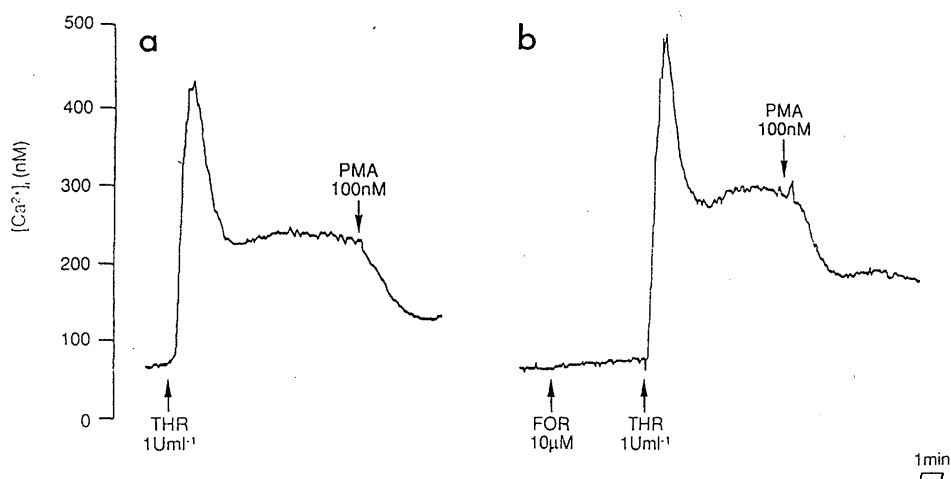


Figure 4.49 Trace (a) illustrates the effects of 4 β -phorbol 12-myristate 13-acetate (PMA; 100nM) when added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (THR; 1U ml⁻¹) in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium. Trace (b) illustrates the effects of pre-treatment with forskolin (FOR; 10 μ M, 5min before thrombin addition) on the PMA-induced fall in $[Ca^{2+}]_i$. Traces (a) and (b) are each representative of 5 individual experiments.

Endothelial cyclic GMP levels rise as a consequence of the EDRF that they produce (Martin et al., 1988) as well as to exogenously added atrial natriuretic factors (Leitman & Murad, 1986; Martin et al., 1988). It has been proposed that the resulting elevations of endothelial cyclic GMP levels inhibit EDRF production (Evans et al., 1988; Hogan et al., 1989). Although the thrombin-induced production of inositol 1, 4, 5-trisphosphate in pig aortic endothelial cells is inhibited by the elevation of cyclic GMP (Lang & Lewis, 1991a), it is not clear whether endothelial calcium mobilisation is inhibited. Therefore, an attempt was made to determine whether or not elevation of cyclic GMP levels modulates calcium mobilisation.

4.6.2 Effects of 8 bromo cyclic GMP and sodium nitroprusside on thrombin-induced elevations of $[Ca^{2+}]_i$ in BAEC

In the presence of 1.8mM extracellular calcium, pre-treatment with 8 bromo cyclic GMP (30 μ M, 5min), a membrane-permeant analogue of cyclic GMP, had no effect on basal $[Ca^{2+}]_i$ or on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (1U ml⁻¹; Figure 4.50).

Neither 8 bromo cyclic GMP (30 μ M) nor sodium nitroprusside (1 μ M), an activator of soluble guanylate cyclase, had any effect on the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (1U ml⁻¹) (n=3 for each; Figure 4.51).

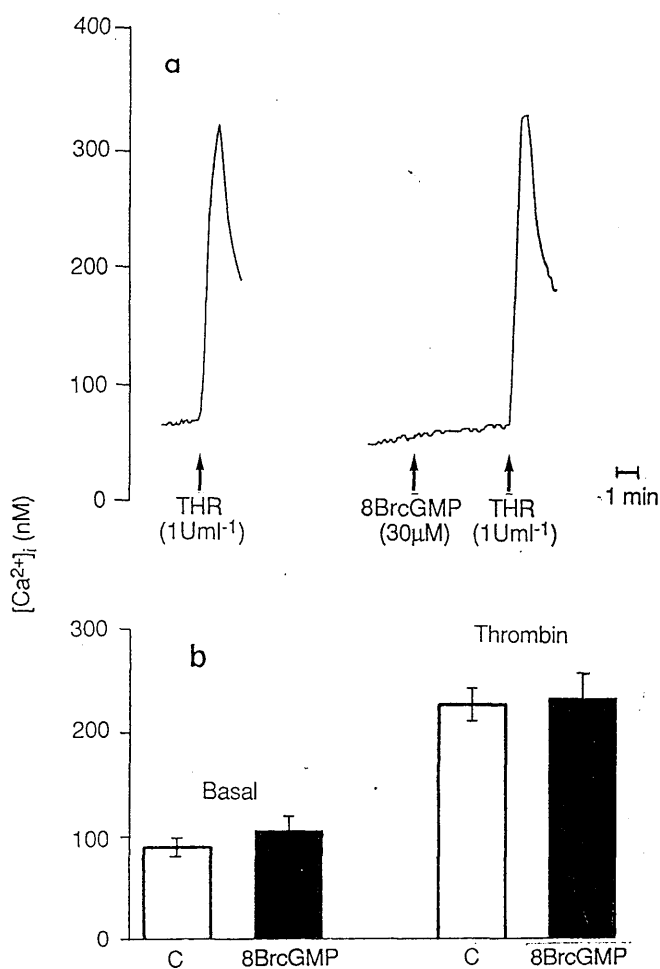


Figure 4.50 Individual traces (a) and a histogram (b) illustrating the effects of pre-treatment with 8 bromo cyclic GMP (8BrcGMP; 30 μM, 5min) on basal levels of $[Ca^{2+}]_i$ and on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (THR; 1U ml⁻¹) in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium. In the histogram, basal levels and the magnitude of the thrombin-induced transient elevations of $[Ca^{2+}]_i$ are presented as the mean of 6-15 observations and vertical bars indicate s.e. mean.

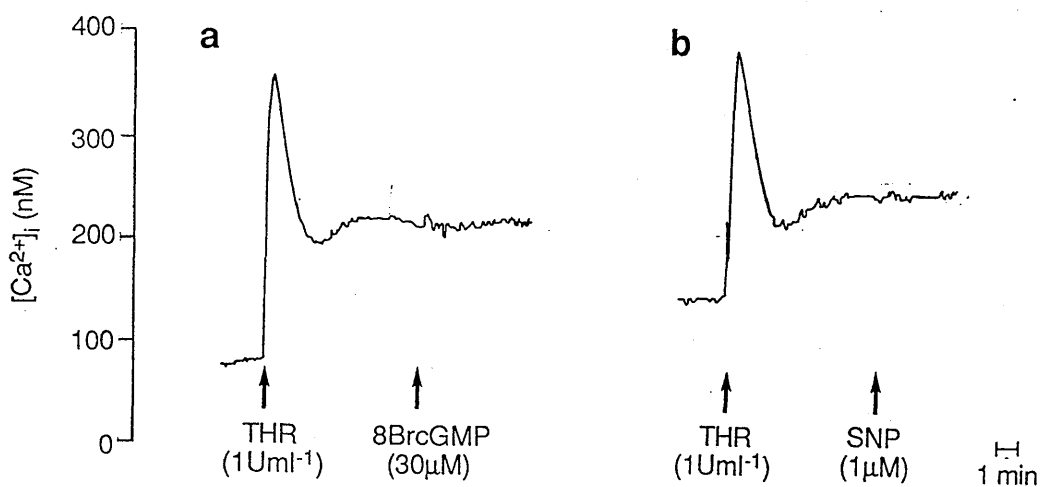


Figure 4.51 Individual traces illustrating the effects of 8 bromo cyclic GMP (a; 8BrcGMP; 30μM) and sodium nitroprusside (b; SNP; 1μM) on the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (THR; 1U ml⁻¹) in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium. Traces (a) and (b) are each representative of 3 separate experiments.

4.6.3 Effects of 8 bromo cyclic GMP and sodium nitroprusside on bradykinin-induced elevations of $[Ca^{2+}]_i$ in BAEC

In the presence of 1.8mM extracellular calcium, pre-treatment with 8 bromo cyclic GMP (30 μ M, 5min) had no effect on basal $[Ca^{2+}]_i$ or on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by bradykinin (10nM; Figure 4.52).

Neither 8 bromo cyclic GMP (30 μ M) nor sodium nitroprusside (1 μ M) had any effect on the plateau phase of the increase in $[Ca^{2+}]_i$ induced by bradykinin (10nM) (n=5 and n=2, respectively; Figure 4.53).

4.6.4 Effects of 8 bromo cyclic GMP and sodium nitroprusside on ATP-induced elevations of $[Ca^{2+}]_i$ in BAEC

In the presence of 1.8mM extracellular calcium, pre-treatment with 8 bromo cyclic GMP (30 μ M, 5min) had no effect on basal $[Ca^{2+}]_i$ or the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by ATP (3 μ M; Figure 4.54).

Neither 8 bromo cyclic GMP (30 μ M) nor sodium nitroprusside (1 μ M) had any effect when added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by ATP (30 μ M) (n=2 for each; Figure 4.55).

4.6.5 Effects on N^G -nitro-L-arginine on $[Ca^{2+}]_i$ in BAEC

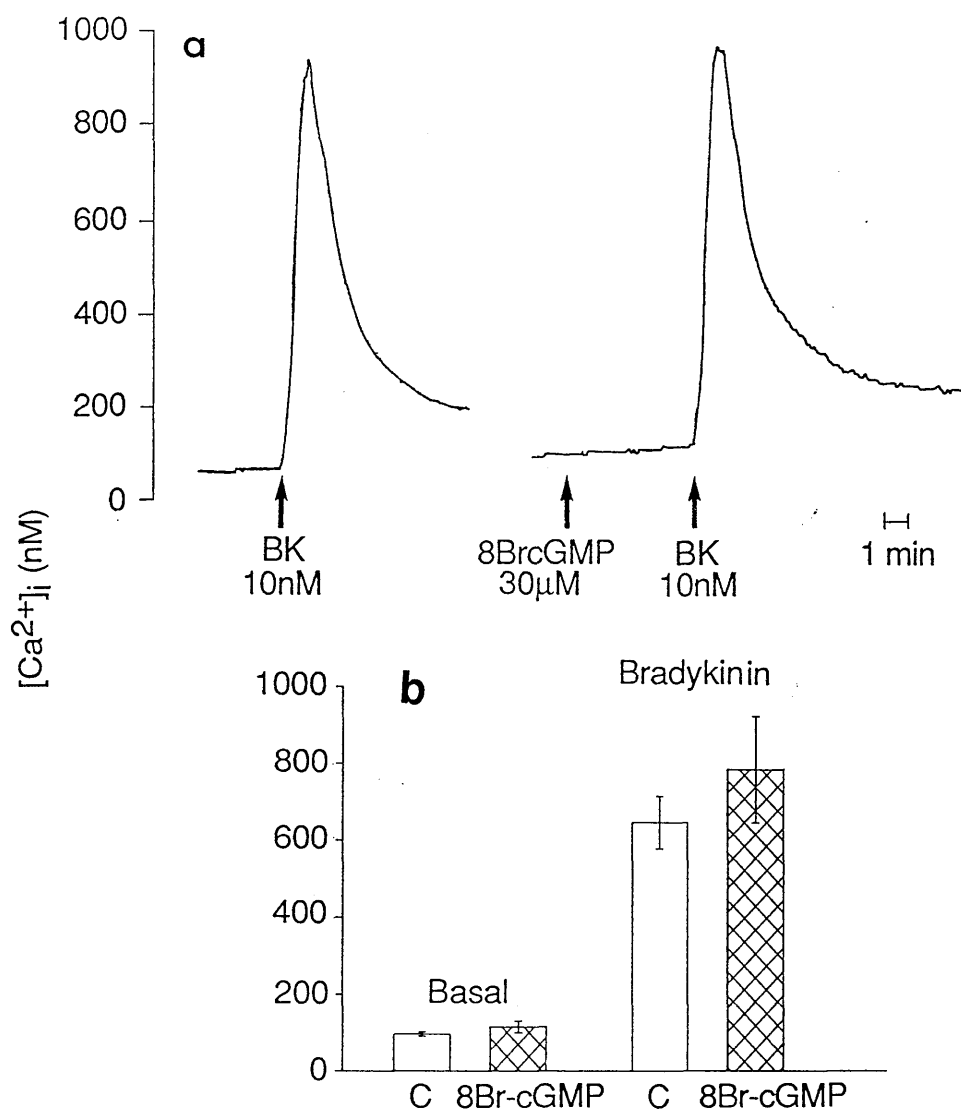


Figure 4.52 Individual traces (a) and a histogram (b) illustrating the effects of pre-treatment with 8 bromo cyclic GMP (8Br-cGMP; 30μM, 5min) on basal levels of $[Ca^{2+}]_i$ and on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by bradykinin (BK; 10nM) in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium. In the histogram, basal levels and the magnitude of the bradykinin-induced transient elevation of $[Ca^{2+}]_i$ are presented as the mean of 4-12 observations and vertical bars indicate s.e. mean.

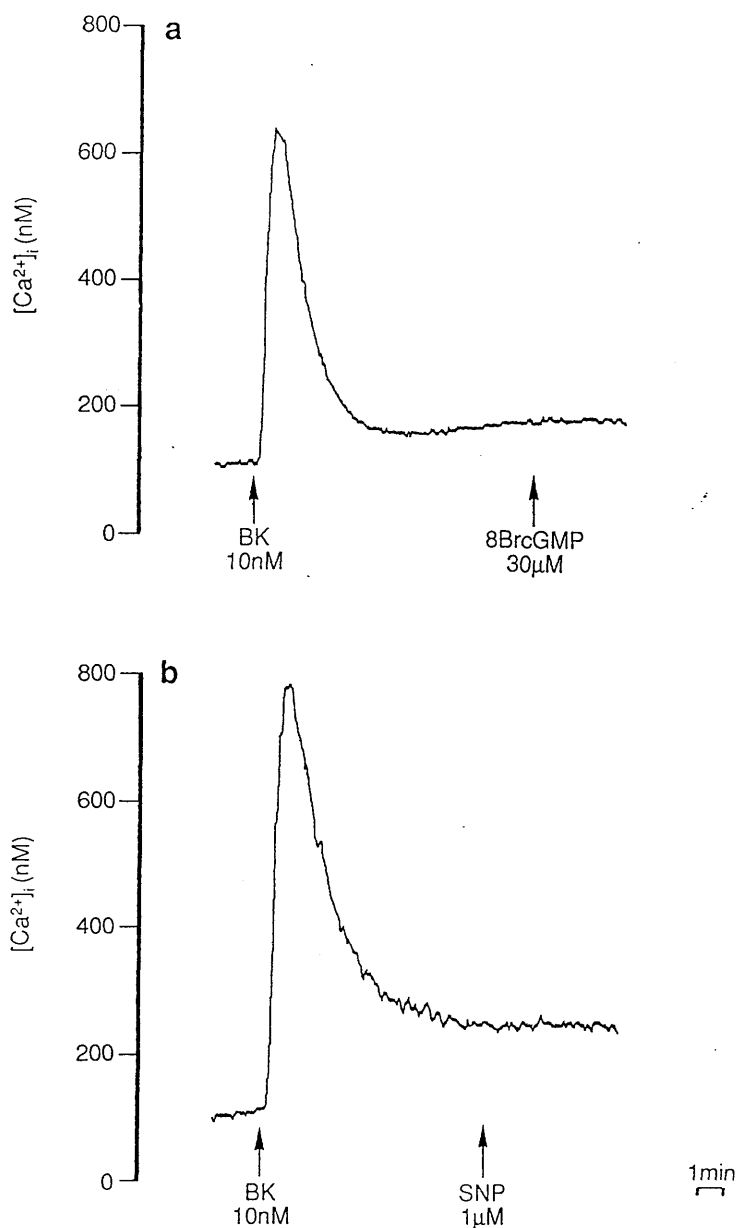


Figure 4.53 Individual traces illustrating the effects of 8 bromo cyclic GMP (a; 8Br cGMP; 30 μ M) and sodium nitroprusside (b; SNP; 1 μ M) on the plateau phase of the increase in $[Ca^{2+}]_i$ induced by bradykinin (BK; 10 nM) in bovine aortic endothelial cells, in the presence of 1.8 mM extracellular calcium. Traces (a) and (b) are representative of 5 and 2 separate experiments, respectively.

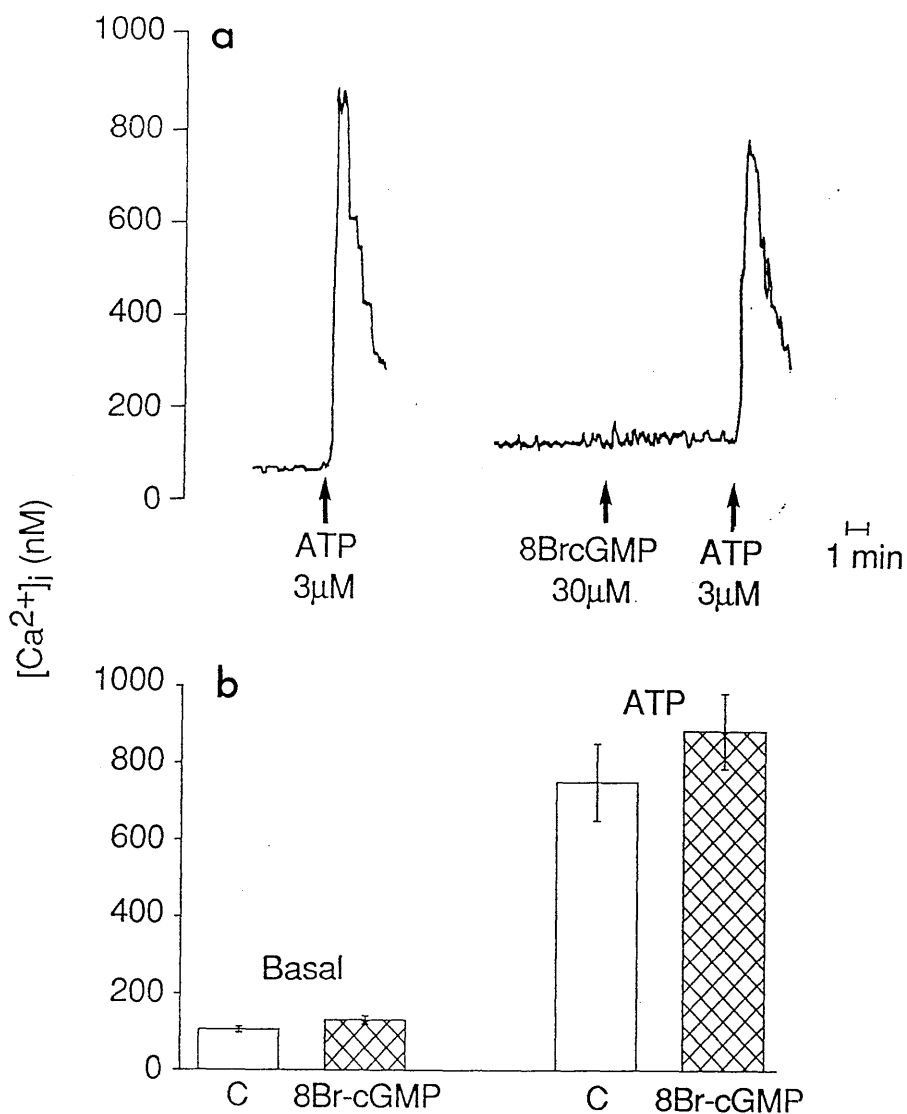


Figure 4.54 Individual traces (a) and a histogram (b) illustrating the effects of pre-treatment with 8 bromo cyclic GMP (8BrcGMP; 30 μ M, 5min) on basal levels of $[Ca^{2+}]_i$ and on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by ATP (3 μ M) in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium. In the histogram, basal levels and the magnitude of the ATP-induced transient elevation of $[Ca^{2+}]_i$ are presented as the mean of 4-9 observations and vertical bars indicate the s.e. mean.

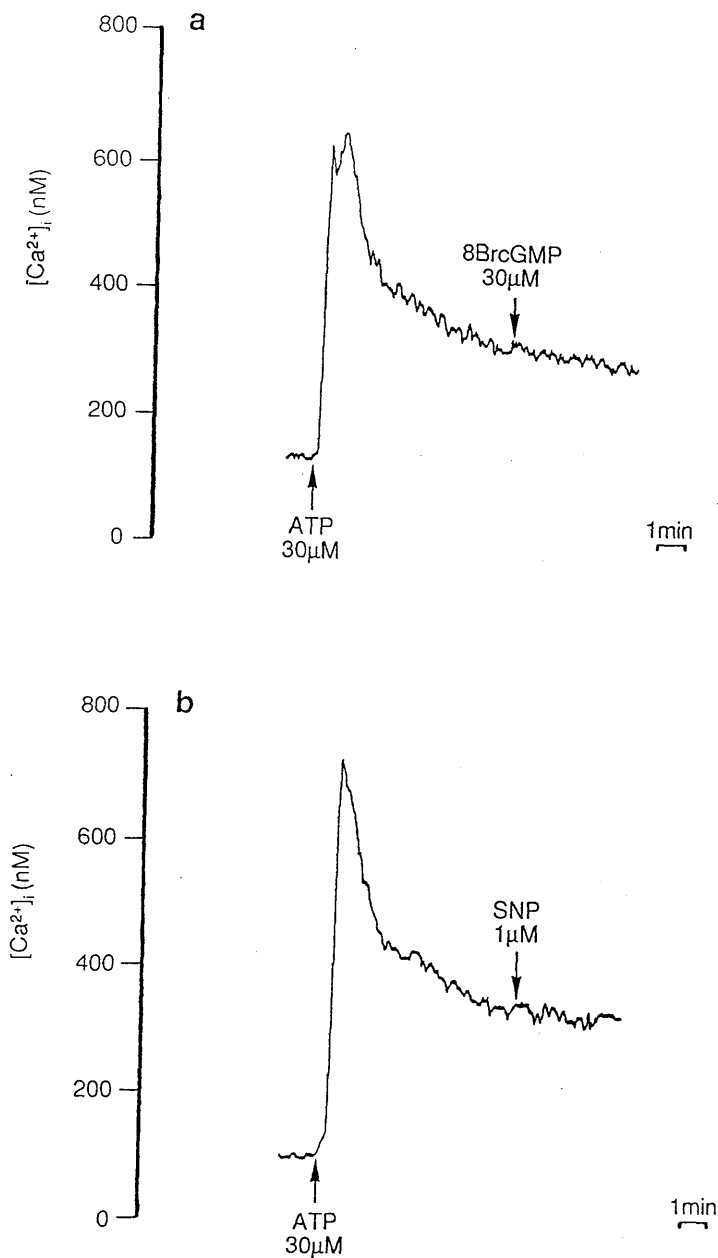


Figure 4.55 Individual traces illustrating the effects of 8 bromo cyclic GMP (a; 8BrcGMP; 30 μ M) and sodium nitroprusside (b; SNP; 1 μ M) on the plateau phase of the increase in $[Ca^{2+}]_i$ induced by ATP (30 μ M) in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium. Traces (a) and (b) are each representative of 2 separate experiments.

Endothelial cyclic GMP content may be elevated in response to agonist-induced production of EDRF (Martin et al., 1988). Therefore, it is possible that endogenously generated cyclic GMP had maximally affected endothelial calcium mobilisation such that further modulation by exogenously added 8 bromo cyclic GMP was not possible. To examine this possibility, endothelial cells were treated with N^G -nitro-L-arginine (L-NOARG), an inhibitor of EDRF production (Moore et al., 1990), and the effect on endothelial calcium mobilisation determined.

In the presence of 1.8mM extracellular calcium, L-NOARG (50 μ M) had no effect on basal levels of $[Ca^{2+}]_i$ or the magnitude of the initial transient elevations of $[Ca^{2+}]_i$ induced by thrombin (1U ml⁻¹), bradykinin (1nM) or ATP (3 μ M) (Figures 4.56, 4.57 and 4.58). Furthermore, L-NOARG (50 μ M) had no effect when added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (1U ml⁻¹; n=3), bradykinin (10nM; n=4) or ATP (30 μ M; n=1) (Figure 4.59).

4.7 Guanine Nucleotide-Dependent Regulatory Proteins (G proteins)

4.7.1 Role of G-proteins in the regulation of $[Ca^{2+}]_i$ in BAEC

Evidence that membrane receptors and intracellular signalling pathways may communicate through a family of transducing proteins, termed guanine nucleotide-dependent regulatory proteins or G-proteins, was first provided by

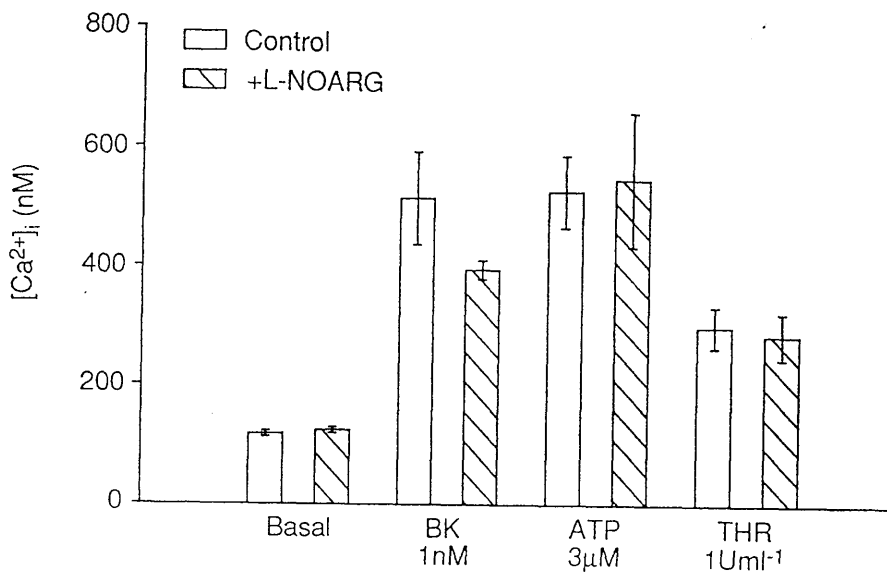


Figure 4.56 Histogram illustrating the effects of pre-treatment with N^G-nitro-L-arginine (L-NOARG; 50 μ M, 5min) on basal $[Ca^{2+}]_i$ and the magnitude of the peak elevation of $[Ca^{2+}]_i$ induced by bradykinin (BK; 1nM), ATP (3 μ M) and thrombin (THR; 1U ml $^{-1}$) in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium. Basal levels and the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ are given as the mean of 6-36 observations and vertical bars indicate s.e. mean.

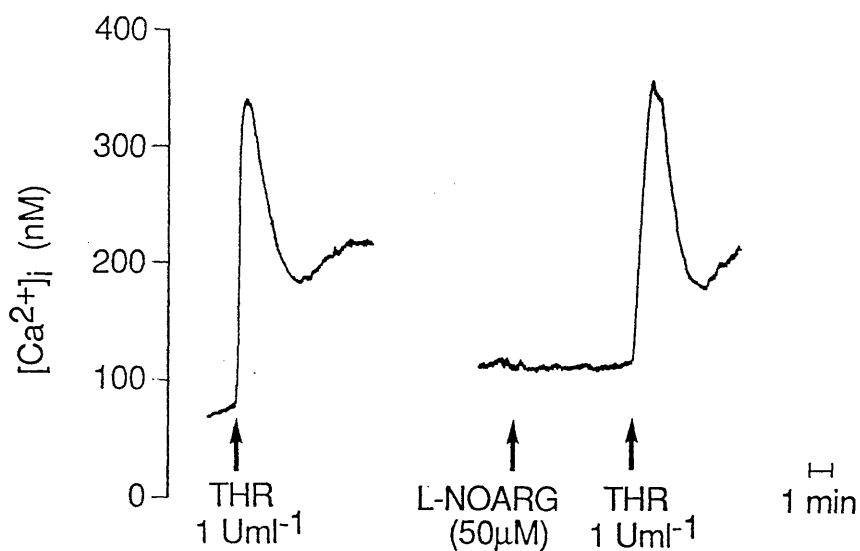


Figure 4.57 Individual traces illustrating the effects of pre-treatment with N^G -nitro-L-arginine (L-NOARG; 50 μM, 5min) on basal levels of $[Ca^{2+}]_i$ and on the magnitude of the initial transient elevations of $[Ca^{2+}]_i$ induced by thrombin (THR; 1U ml⁻¹) in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium.

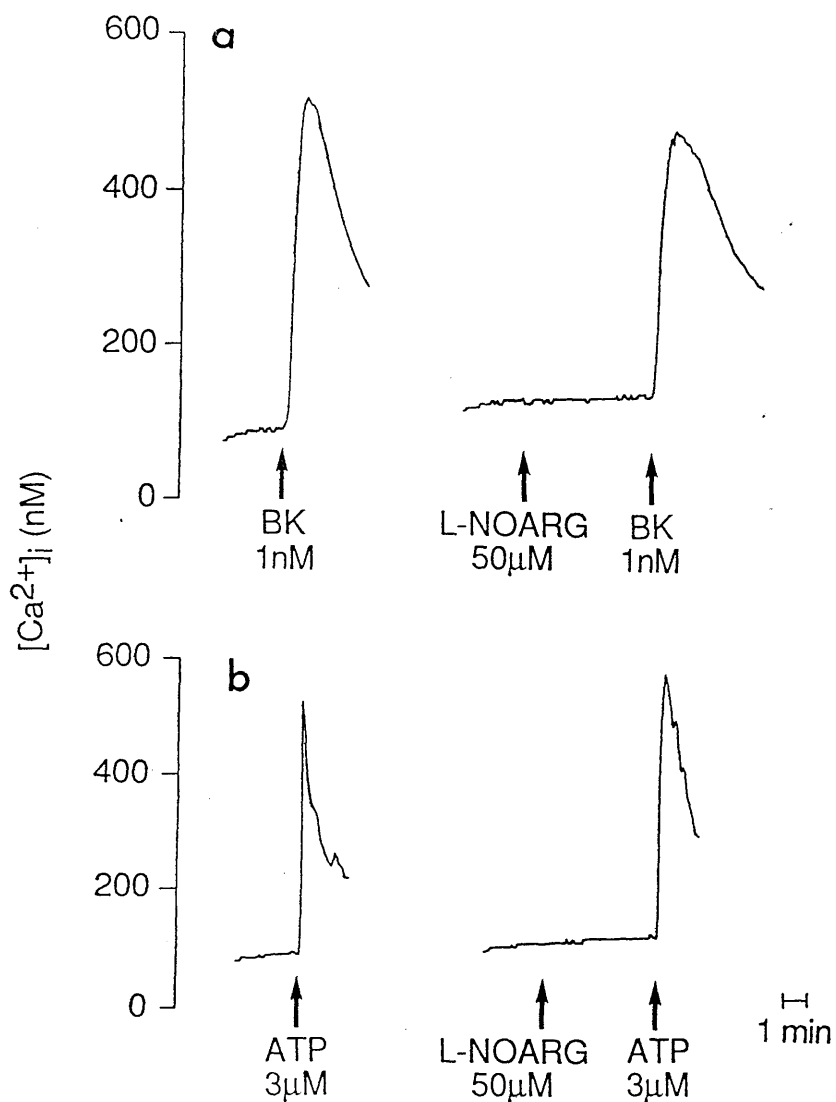


Figure 4.58 Individual traces illustrating the effects of pre-treatment with N^G -nitro-L-arginine (L-NOARG; 50 μ M, 5min) on basal levels of $[Ca^{2+}]_i$ and on the magnitude of the initial transient elevations of $[Ca^{2+}]_i$ induced by bradykinin (a; BK; 1nM) and ATP (b; 3 μ M) in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium.

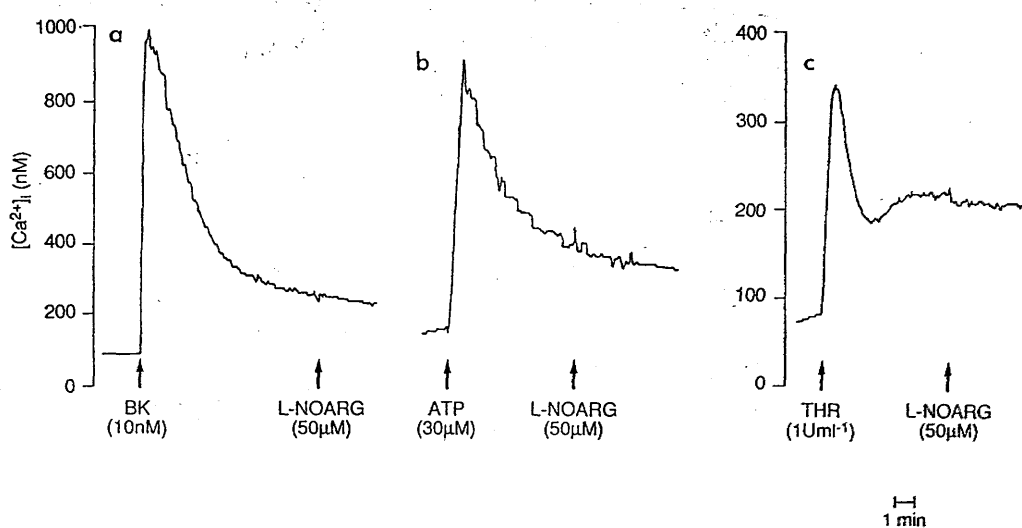


Figure 4.59 Individual traces illustrating the effects of addition of N^G -nitro-L-arginine (L-NOARG; $50\mu M$) during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by bradykinin (BK; $10nM$), ATP ($30\mu M$) and thrombin (THR; $1U ml^{-1}$) in bovine aortic endothelial cells, in the presence of $1.8mM$ extracellular calcium. The traces (a), (b) and (c) are representative of 4, 1 and 3 experiments, respectively.

Rodbell et al. (1971). Recent studies suggest that the phosphoinositidase C-mediated hydrolysis of phosphatidylinositol 4, 5 - biphosphate ($\text{PtdIns}(4,5)\text{P}_2$) may be regulated via a G protein (Cockcroft & Stutchfield 1988). Furthermore, it has been proposed that the ATP-induced hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ and subsequent mobilisation of calcium in bovine aortic endothelial cells is regulated by a G-protein (Brock et al., 1988). G-proteins are often inhibited by bacterial toxins (for example, pertussis toxin), although susceptibility to the actions of these toxins may even vary between different agonists in a single cell type, as observed by Brass et al. (1987) in the platelet. We wished to determine whether calcium mobilisation induced by thrombin, bradykinin and ATP in bovine aortic endothelial cells is under the influence of pertussis toxin-sensitive G proteins.

4.7.2 Effects of pertussis toxin on $[\text{Ca}^{2+}]_i$ in BAEC

Monolayers of BAEC were incubated for 20hrs in complete medium containing pertussis toxin (25ng ml^{-1}). Control monolayers were transferred to fresh medium for an identical period. At the end of this period, endothelial monolayers were loaded with fura-2 followed by measurement of $[\text{Ca}^{2+}]_i$.

Generally, treatment with pertussis toxin had no effect on basal levels of $[\text{Ca}^{2+}]_i$ in BAEC (Figures 4.60 and 4.62), although in some batches of cells, basal levels of $[\text{Ca}^{2+}]_i$

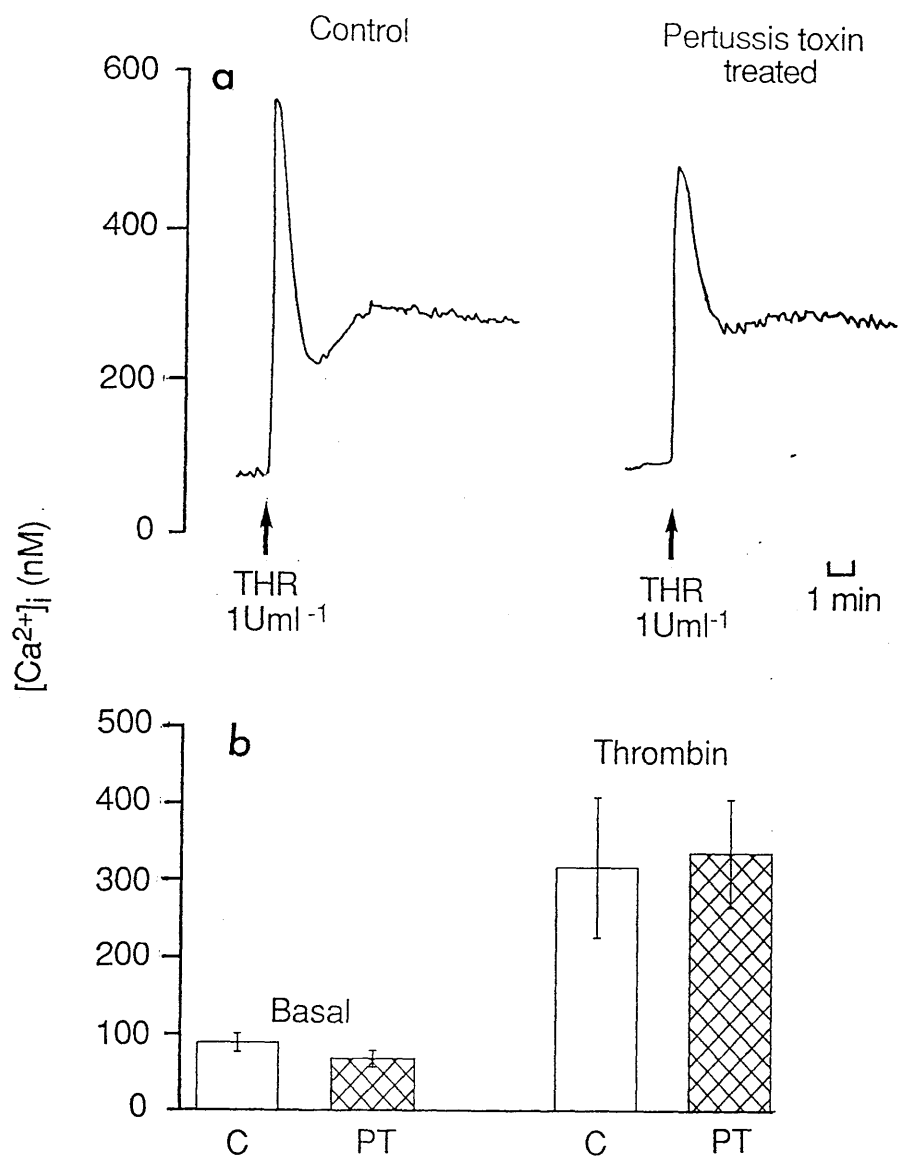


Figure 4.60 Individual traces (a) and a histogram (b) illustrating the effects of pre-treatment with pertussis toxin (PT; $25 ng ml^{-1}$, 20hr) on basal and thrombin (THR; $1 U ml^{-1}$) - stimulated levels of $[Ca^{2+}]_i$ in bovine aortic endothelial cells, in the presence of $1.8 mM$ extracellular calcium. In the histogram, basal levels and the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ in control cells (open columns) and pertussis toxin treated cells (cross-hatched columns) are given as the mean of the 4 observations and vertical bars indicate the s.e. mean.

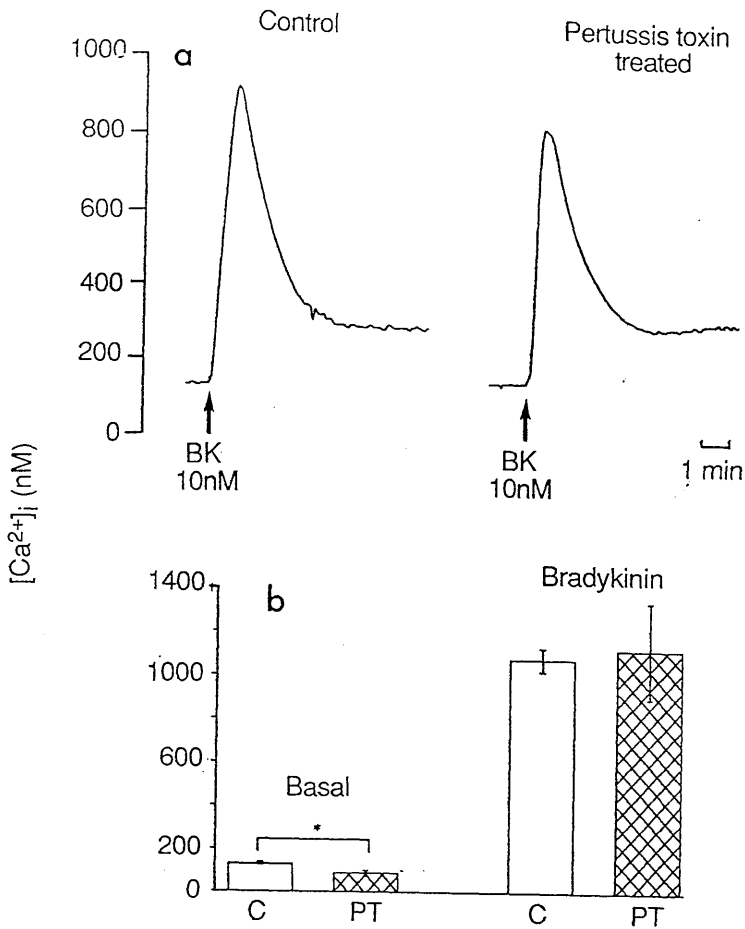


Figure 4.61 Individual traces (a) and a histogram (b) illustrating the effects of pre-treatment with pertussis toxin (PT; 25ng ml^{-1} , 20hr) on basal and bradykinin (BK; 10nM) - stimulated levels of $[Ca^{2+}]_i$ in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium. In the histogram, basal levels and the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ in control cells (open columns) and pertussis toxin-treated cells (cross-hatched columns) are given as the mean of 4 observations and vertical bars indicate the s.e. mean. * $p < 0.05$ indicates a significant difference between groups joined by a bracket.

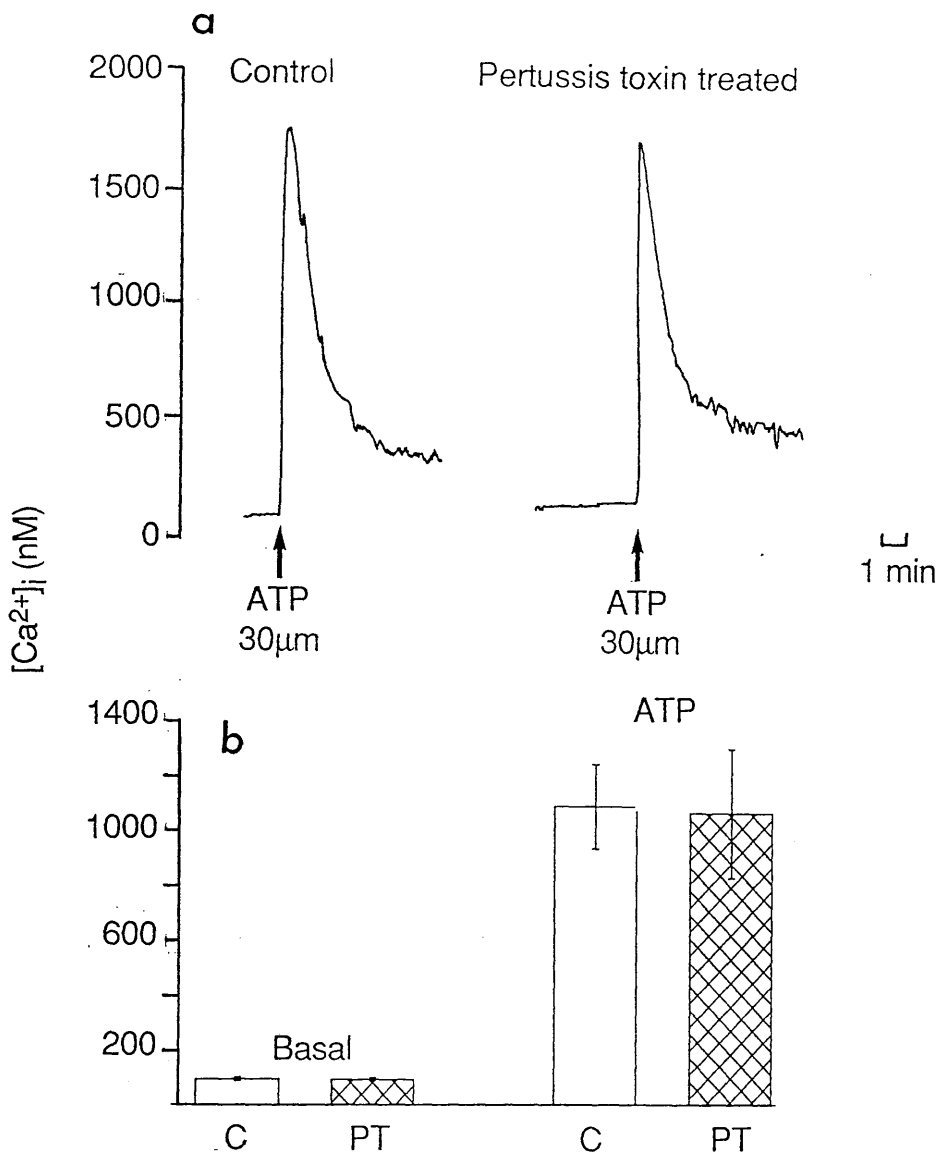


Figure 4.62 Individual traces (a) and a histogram (b) illustrating the effects of pre-treatment with pertussis toxin (PT; 25ng ml⁻¹, 20hr) on basal and ATP (30μM) - stimulated levels of [Ca²⁺]_i in bovine aortic endothelial cells, in the presence of 1.8mM extracellular calcium. In the histogram, basal levels and the magnitude of the initial transient elevation of [Ca²⁺]_i in control cells (open columns) and pertussis toxin-treated cells (cross-hatched columns) are given as the mean of 8 observations and vertical bars illustrate the s.e. mean.

were found to be slightly, but significantly, reduced (Figure 4.61).

However, treatment with pertussis toxin had no effect on either the initial transient or the plateau phase of the increases in $[Ca^{2+}]_i$ induced by thrombin ($1U\ ml^{-1}$; Figure 4.60), bradykinin ($10nM$; Figure 4.61) or ATP ($30\mu M$; Figure 4.62).

5.1.1 Role of the endothelial cell in the production of increased vascular permeability

Majno and Palade (1961) proposed that the ability of histamine to induce increases in vascular permeability resulted from active endothelial cell contraction and the subsequent formation of inter-endothelial cell gaps. Several difficulties are associated with evaluating the role of the endothelial cell in changes in vascular permeability in vivo; these include technical difficulties associated with changes in blood flow or hydrostatic gradients, the interaction of endothelial cells with other cell types (for example, white blood cells) and in determining experimental drug concentrations precisely. Many of these problems may be overcome by the use of an in vitro system, in which drug concentrations can be accurately controlled and effects on pure homogenous populations of endothelial cells can be evaluated. Various in vitro systems, in which endothelial cells are grown on membrane filters, have been described previously (Shasby et al., 1982, 1985; Rotrosen & Gallin, 1986; Gudgeon & Martin, 1989). These endothelial cells, grown on membranes, maintain a profile of barrier function which is similar to that obtained in vivo (Del-Vecchio et al., 1987). It is predominantly the endothelial cells of the post-capillary venule which are involved in the permeability changes occurring during inflammatory oedema (Majno et al., 1961; Svensjö et al., 1979). These cells are difficult to isolate

successfully, but various endothelial cell types with a similar profile of responsiveness can be isolated : these include human umbilical vein endothelial cells or bovine pulmonary artery endothelial cells (Rotrosen & Gallin, 1986; Casnocha et al., 1989; Lum et al., 1989; Minnear et al., 1989).

5.1.2 Agents which inhibit endothelial barrier function :

4 β -phorbol 12-myristate 13-acetate and thrombin

Previous studies have indicated that, in the absence of endothelial cells, transfer of trypan blue-labelled albumin (4%) across Transwell polycarbonate membrane filters occurs rapidly (Gudgeon & Martin, 1989). The presence of cultured endothelial cells restricts this movement (Figure 5.1). In this study, BPAEC were found to restrict the movement of trypan blue-labelled albumin into the lower chamber. After 90 min, resting levels (control) of transfer of albumin were typically found to be 2-10% (Figures 5.2-5.7) (complete equilibration = 100%).

Addition of 4 β -phorbol 12-myristate 13-acetate (PMA, 600nM), an activator of protein kinase C, to BPAEC monolayers resulted in a 2-3 fold increase in the transfer of albumin across the monolayers after 90 min (Figures 5.2, 5.4 and 5.6). Thrombin (1U ml⁻¹) was also found to induce a 1.5-2 fold increase in albumin transfer after a 90 min incubation (Figures 5.3, 5.5 and 5.7).

5.2.1 Agents which enhance endothelial barrier function

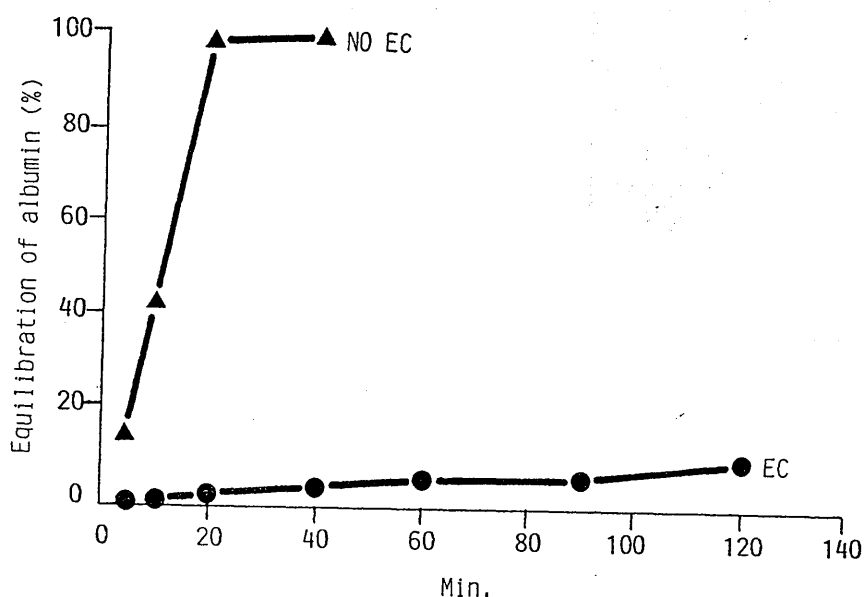


Figure 5.1 Time course showing the passage of trypan blue-labelled albumin through Transwell filters in the presence (EC) and absence (no EC) of pig aortic endothelial cells. HEPES (5mM)-buffered Krebs containing 4% trypan blue-labelled albumin was placed in the upper chamber and its passage into the lower chamber, which contained only HEPES-buffered Krebs, measured at various time points. Full equilibration between the upper and lower chambers was 100%. Modified from Gudgeon and Martin (1989).

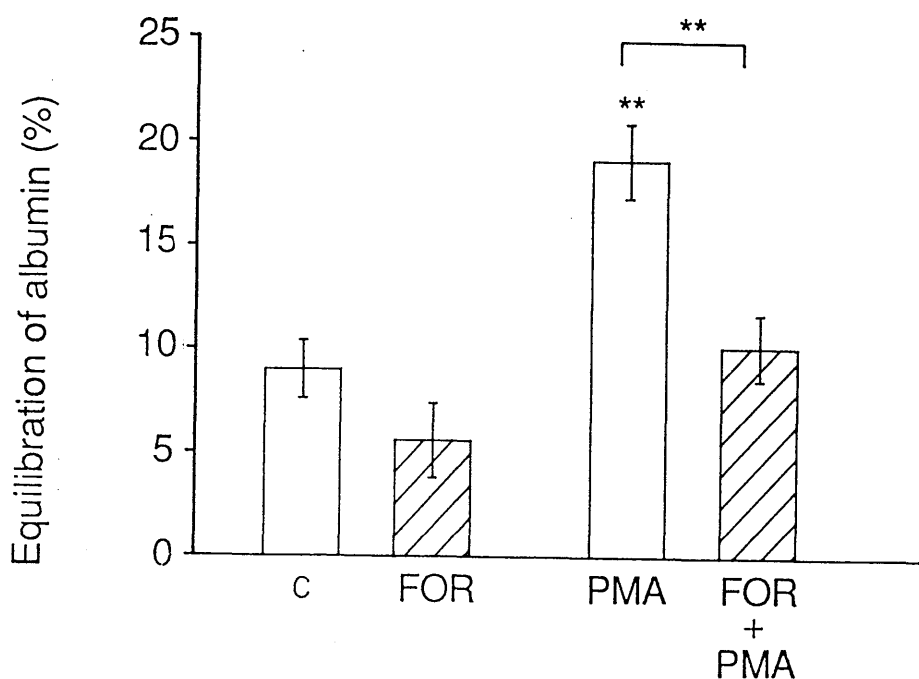


Figure 5.2 Histogram showing the effects of forskolin (FOR; 30 μ M, 90min) on control (C) and 4 β -phorbol 12-myristate 13-acetate (PMA; 600nM, 90min) - stimulated transfer of trypan blue-labelled albumin across monolayers of bovine pulmonary artery endothelial cells. Levels of equilibration of albumin are given as the mean of 6 observations and vertical bars indicate the s.e. mean. ** $p < 0.01$ denotes a significant difference from levels of albumin transfer across control monolayers. A significant difference ($p < 0.01$) exists between groups joined by a bracket.

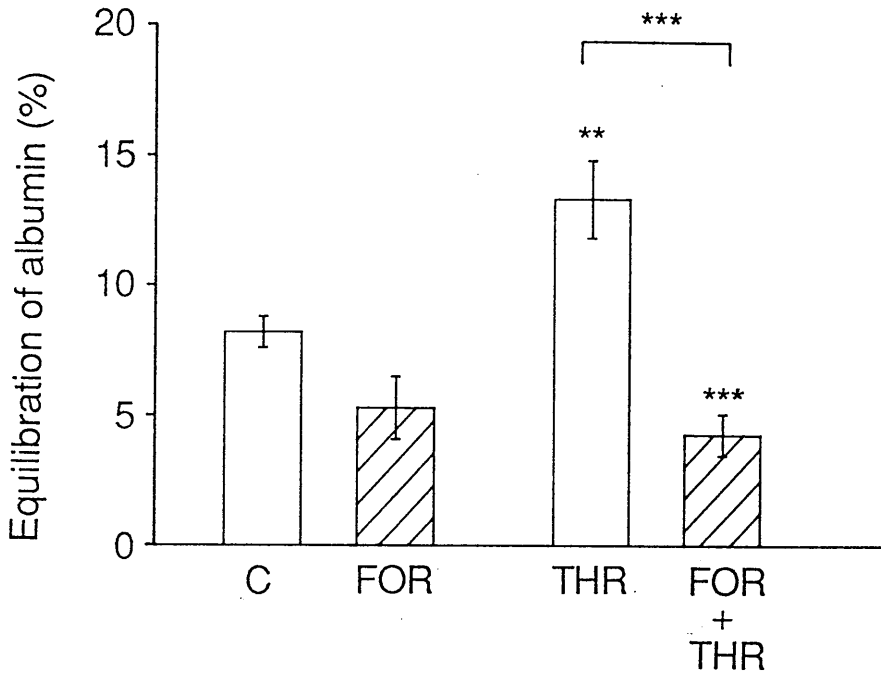


Figure 5.3 Histogram demonstrating the effects of forskolin (FOR; $30\mu\text{M}$, 90min) on control (C) and thrombin (THR; 1U ml^{-1} , 90min) - stimulated transfer of trypan blue-labelled albumin across monolayers of bovine pulmonary artery endothelial cells. Levels of equilibration of albumin are given as the mean of 12 observations and vertical bars indicate the s.e. mean. ** $p < 0.01$, *** $p < 0.001$ denotes a significant difference from levels of albumin transfer across control monolayers. A significant difference ($p < 0.001$) exists between groups joined by a bracket.

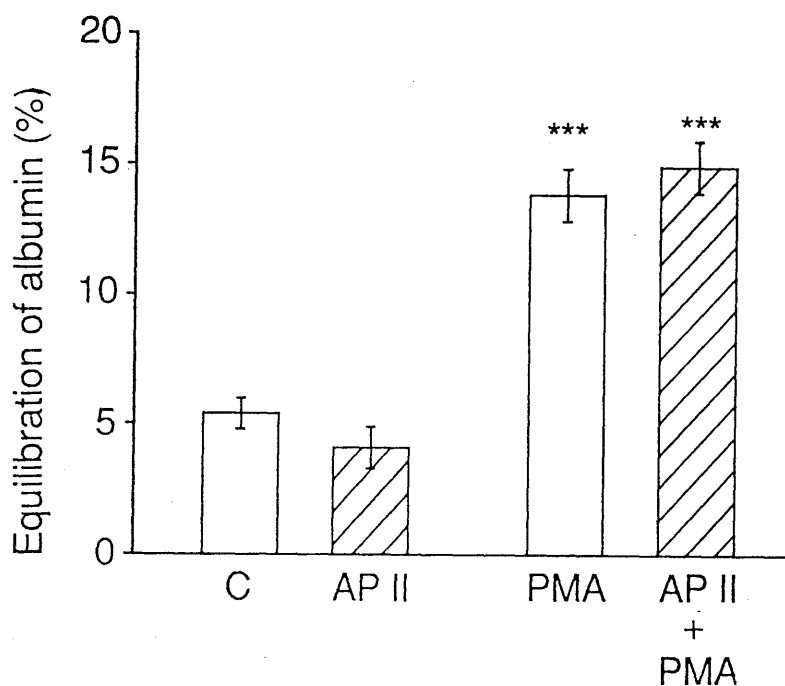


Figure 5.4 Histogram demonstrating the effects of atriopeptin II (APII; 100nM, 90min) on basal and 4 β -phorbol 12-myristate 13-acetate (PMA; 600nM, 90min) - stimulated transfer of trypan blue-labelled albumin across monolayers of bovine pulmonary artery endothelial cells. Levels of equilibration of albumin are given as the mean of 6 observations and vertical bars indicate the s.e. mean. ***p<0.001 denotes a significant difference from levels of albumin transfer across control monolayers.

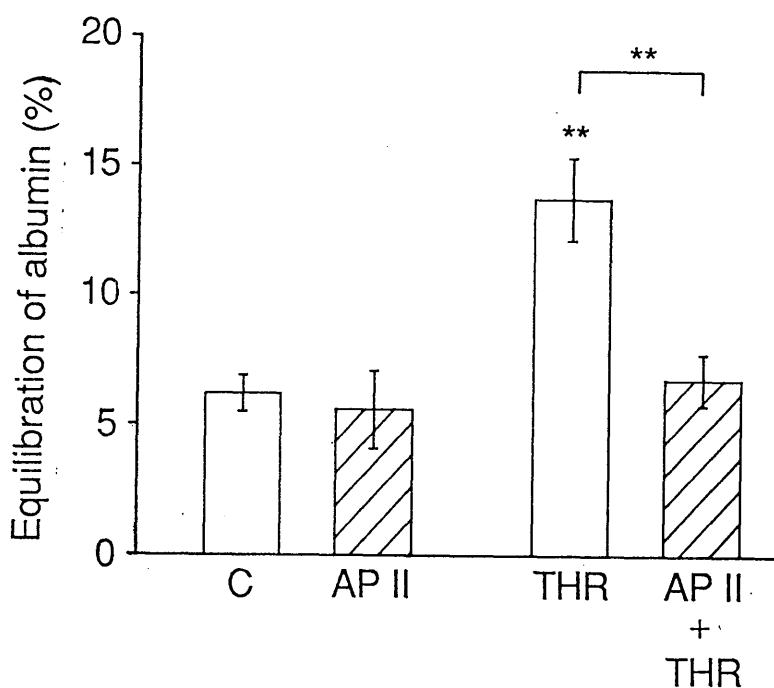


Figure 5.5 Histogram demonstrating the effects of atriopeptin II (APII; 100nM, 90min) on control (C) and thrombin (THR; 1U ml⁻¹, 90min) - stimulated transfer of trypan blue-labelled albumin across monolayers of bovine pulmonary artery endothelial cells. Levels of equilibration of albumin are given as the mean of 6 observations and vertical bars indicate the s.e. mean. **p<0.01 denotes a significant difference from levels of albumin transfer across control monolayers. A significant difference (p<0.01) exists between groups joined by a bracket.

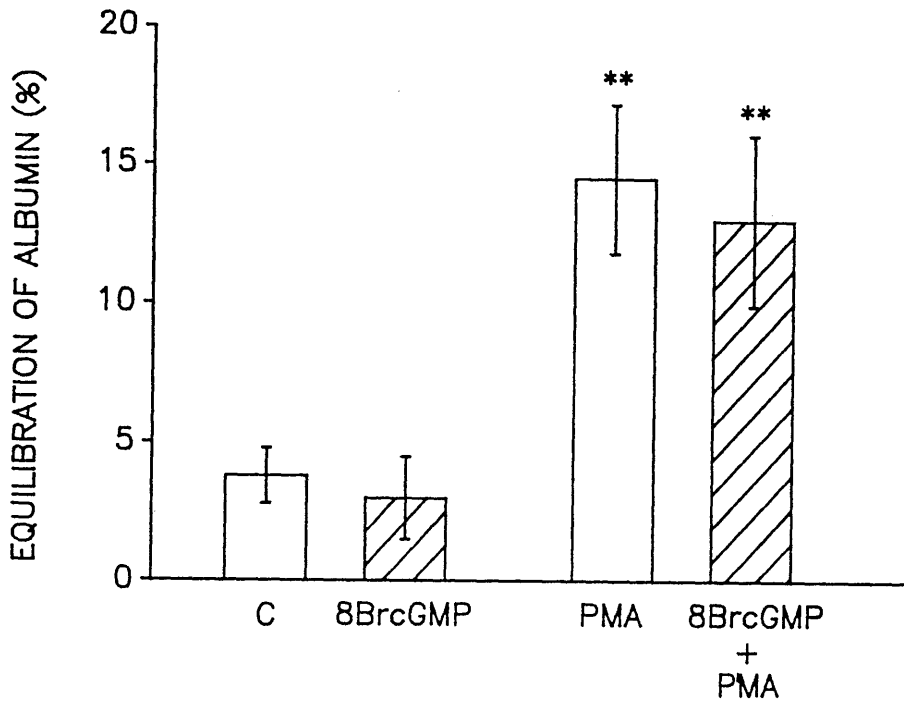


Figure 5.6 Histogram illustrating the effects of 8 bromo cyclic GMP (8BrcGMP; $30\mu\text{M}$, 90min) on control (C) and 4 β -phorbol 12-myristate 13-acetate (PMA; 600nM, 90min) - stimulated transfer of trypan blue-labelled albumin across monolayers of bovine pulmonary artery endothelial cells. Levels of equilibration of albumin are given as the mean of 6 observations and vertical bars indicate the s.e. mean. * $p < 0.01$ denotes a significant difference from levels of albumin transfer across control monolayers.

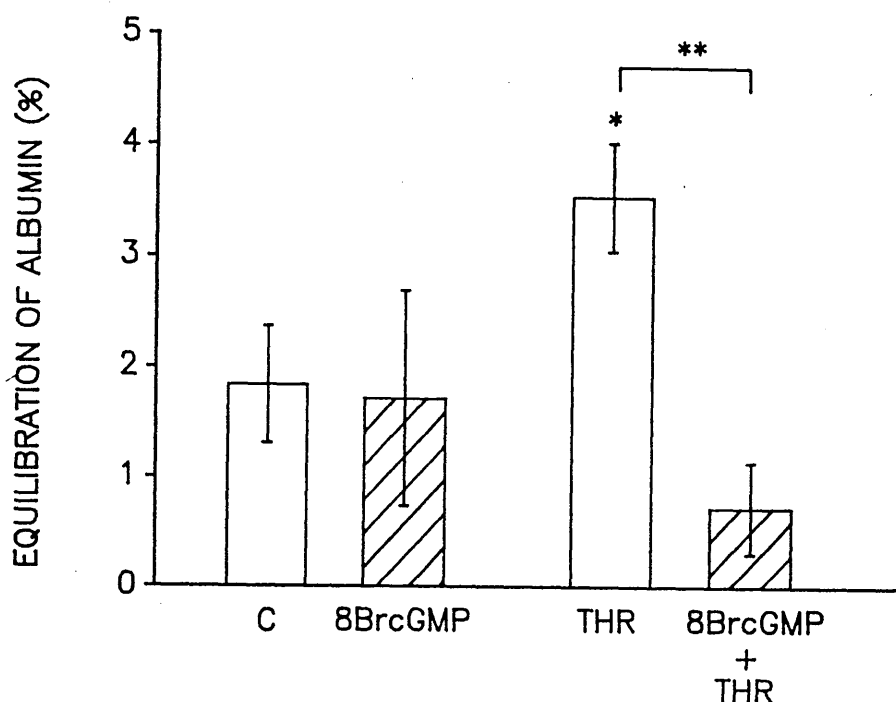


Figure 5.7 Histogram illustrating the effects of 8 bromo cyclic GMP (8BrcGMP; $30\mu\text{M}$, 90min) on control (C) and thrombin (THR; 1U ml^{-1} , 90min) - stimulated transfer of trypan blue-labelled albumin across monolayers of bovine pulmonary artery endothelial cells. Levels of equilibration of albumin are given as the mean of 5-6 observations and vertical bars indicate the s.e. mean. * $p < 0.05$ denotes a significant difference from levels of albumin transfer across control monolayers. A significant difference ($p < 0.01$) exists between groups joined by a bracket.

Recent studies have demonstrated that elevation of cyclic AMP content, but not cyclic GMP content, aids the maintenance of epithelial tight junction integrity (Duffey et al., 1981). This is in contrast to smooth muscle where elevation of cyclic AMP or cyclic GMP content produces relaxation (Itoh et al., 1985). *In vivo*, the ability of β -adrenoceptor agonists to inhibit increases in vascular permeability induced by inflammatory agents is well-documented; this may result from an inhibitory action of inter-endothelial gap formation (Marciniak et al., 1978; Svensjö & Grega, 1986). This inhibitory action of β -adrenoceptor agonists suggests a possible role of cyclic AMP in the maintenance of endothelial barrier function. Therefore, we wished to determine whether elevation of cyclic AMP or cyclic GMP content could enhance the barrier function of bovine pulmonary artery endothelial cells.

5.2.2 Effects of forskolin on the ability of BPAEC to maintain barrier function

Incubation of BPAEC monolayers with forskolin ($30\mu\text{M}$, 90min), a direct activator of adenylate cyclase, had no significant effect on control levels of albumin transfer (Figures 5.2 and 5.3). Co-incubation with forskolin ($30\mu\text{M}$) and PMA (600nM) for 90 min abolished the PMA-induced increase in albumin transfer (Figure 5.2). Co-incubation of forskolin ($30\mu\text{M}$) with thrombin (1U ml^{-1}) for 90 min abolished the thrombin-induced increase in albumin transfer (Figure 5.3), reducing it to slightly below control levels.

5.2.3 Effects of atriopeptin II on the ability of BPAEC to maintain barrier function

The atrial natriuretic factor, atriopeptin II, stimulates particulate guanylate cyclase and elevates endothelial cyclic GMP levels (Leitman & Murad, 1986; Martin et al., 1988). Atriopeptin II (100nM) had no effect on control levels of albumin transfer across BPAEC monolayers following a 90 min incubation (Figures 5.4 and 5.5). Co-incubation with atriopeptin II (100nM) and PMA (600nM) for 90 min had no effect on the increase in albumin transfer induced by PMA (Figure 5.4). In contrast, co-incubation with atriopeptin II (100nM) and thrombin (1U ml⁻¹) for 90 min abolished the thrombin-induced increase in albumin transfer (Figure 5.5).

5.2.4 Effects of 8 bromo cyclic GMP on the ability of BPAEC to maintain barrier function

8 bromo cyclic GMP (8BrcGMP; 30μM), a membrane-permeant analogue of cyclic GMP, had no effect on control levels of albumin transfer across BPAEC monolayers (Figures 5.6 and 5.7). Co-incubation with 8 bromo cyclic GMP (30μM) and PMA (600nM) for 90 min had no effect on the PMA-induced increase in albumin transfer (Figure 5.6). In contrast, co-incubation of 8 bromo cyclic GMP (30μM) with thrombin (1U ml⁻¹) for 90 min abolished the increase in albumin transfer induced by thrombin (Figure 5.7).

5.3.1 Role of protein kinase C in the regulation of [Ca²⁺]_i in BPAEC

It has been proposed that calcium mobilisation plays an important role in the induction of agonist-induced increases in endothelial permeability (Rotrosen & Gallin, 1986; Lum et al., 1989). Supporting the findings of Gudgeon and Martin (1989), this study has demonstrated that activation of protein kinase C, by tumour-promoting phorbol esters, results in an increase in endothelial permeability. The objects of this part of the study were to determine if activation of protein kinase C modulated calcium mobilisation in BPAEC and if changes in calcium mobilisation correlate with changes in endothelial barrier function.

5.3.2 Effects of 4 β -phorbol 12-myristate 13-acetate on thrombin-induced mobilisation of $[Ca^{2+}]_i$ in BPAEC

In the presence of 1.8mM extracellular calcium, 4 β -phorbol 12-myristate 13-acetate (PMA; 100nM, 5min) had no effect on basal levels of $[Ca^{2+}]_i$ in BPAEC (Figure 5.8), but reduced the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (1U ml⁻¹) (Figure 5.8). Addition of PMA (100nM) during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (1U ml⁻¹) resulted in a fall in $[Ca^{2+}]_i$ (Figure 5.9).

5.4.1 Effects of cyclic AMP in the regulation of $[Ca^{2+}]_i$ in BPAEC

In addition to this study, elevation of cyclic AMP content has previously been shown to inhibit increases in endothelial permeability induced by thrombin (Casnocha et

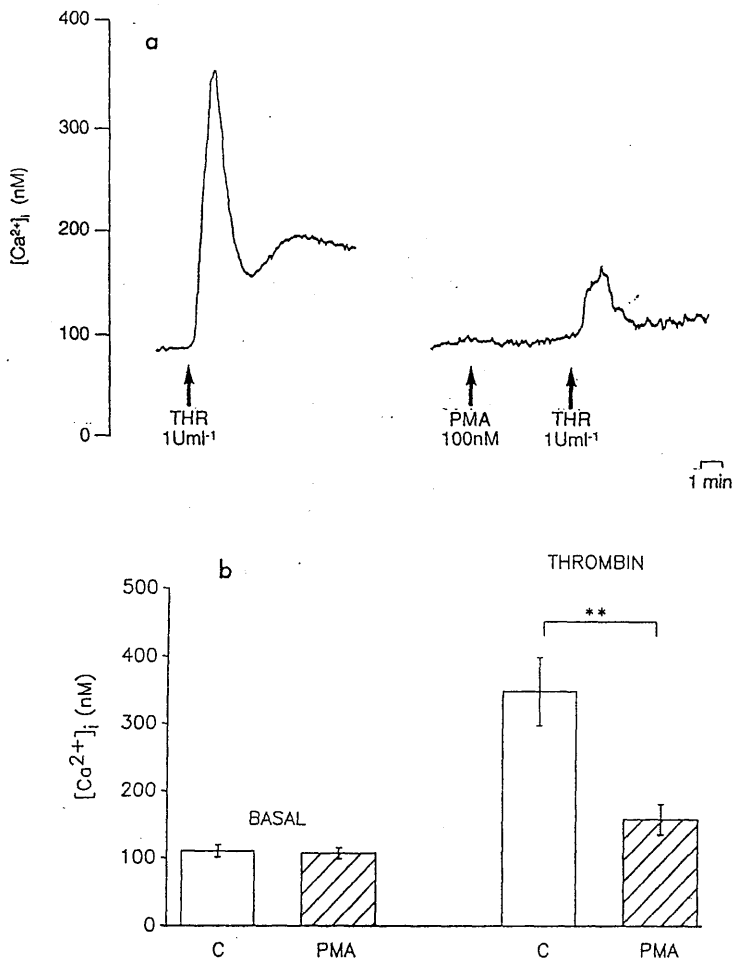


Figure 5.8 Individual traces (a) and a histogram (b) illustrating the effects of pre-treatment with 4 β -phorbol 12-myristate 13-acetate (PMA; 100nM, 5min) on basal levels of $[Ca^{2+}]_i$ and on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (THR; 1U ml⁻¹) in bovine pulmonary artery endothelial cells, in the presence of 1.8mM extracellular calcium. In the histogram, basal and thrombin-induced elevations of $[Ca^{2+}]_i$ in control cells (open columns) and PMA-treated cells (hatched columns) are presented as the mean of 7-16 observations and vertical bars indicate s.e. mean. ***p<0.001 indicates a significant difference between groups joined by a bracket.

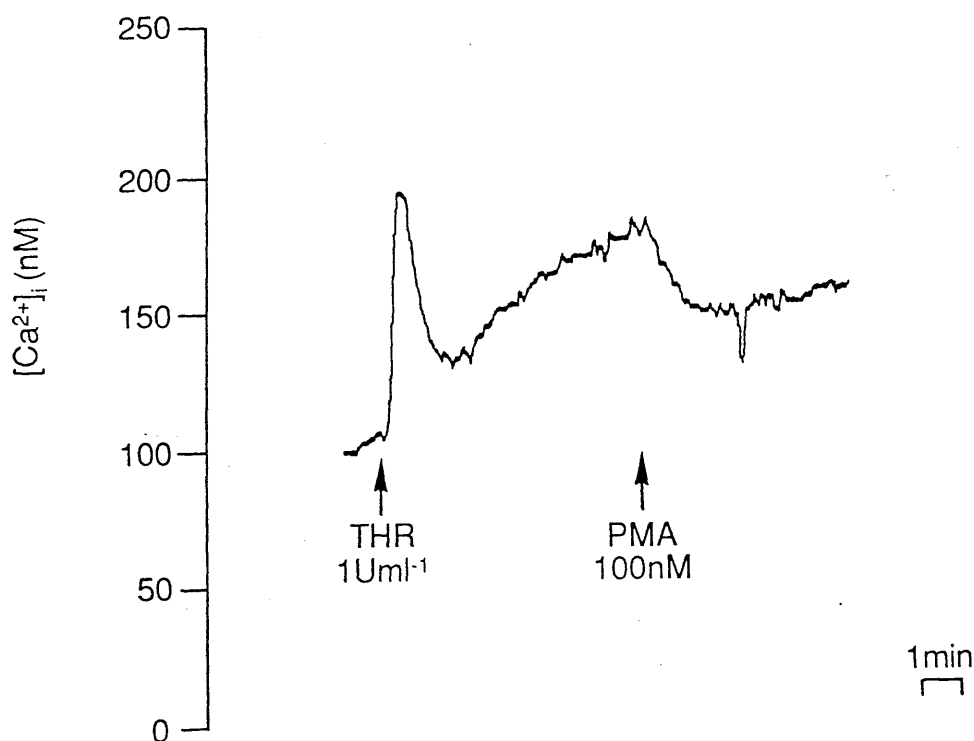


Figure 5.9 Individual traces illustrating the effects of 4 β -phorbol 12-myristate 13-acetate (PMA; 100nM) on the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (THR; 1U ml $^{-1}$; a) in bovine pulmonary artery endothelial cells, in the presence of 1.8mM extracellular calcium. The trace is representative of 2 separate experiments.

al., 1989; Minnear et al., 1989) and histamine (Carson et al., 1989). As a number of studies have proposed that agonist-induced calcium mobilisation may be the trigger for increases in endothelial permeability (Rotrosen & Gallin, 1986; Lum et al., 1989), an attempt was made to determine whether or not cyclic AMP exerted its inhibitory actions on permeability via inhibition of endothelial calcium mobilisation.

5.4.2 Effects of forskolin on thrombin-induced elevations of $[Ca^{2+}]_i$ in BPAEC

In the presence of 1.8mM extracellular calcium, pre-treatment with forskolin (30 μ M, 5min) had no effect on basal levels of $[Ca^{2+}]_i$ or the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (1U ml⁻¹) (Figure 5.10). Addition of forskolin (30 μ M) during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (1U ml⁻¹) resulted in a rapid increase in $[Ca^{2+}]_i$ of 67 \pm 7nM (n=6) (Figure 5.11), which remained relatively stable for at least 5 min.

5.5.1 Effects of cyclic GMP on the regulation of $[Ca^{2+}]_i$ in BPAEC

In this study, elevation of cyclic GMP content by atriopeptin II and 8 bromo cyclic GMP, has been shown to inhibit the thrombin-induced increase in albumin transfer across BPAEC monolayers. An attempt was made to determine whether or not cyclic GMP enhanced barrier function in BPAEC by inhibiting thrombin-induced calcium mobilisation.

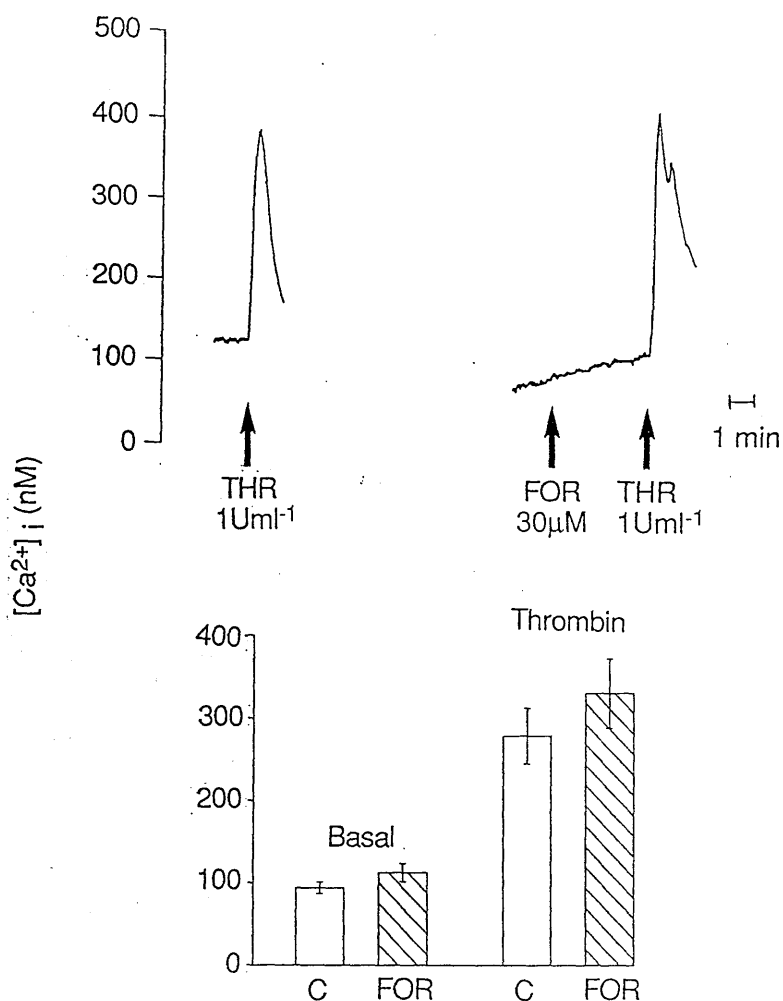


Figure 5.10 Individual traces (a) and a histogram (b) illustrating the effects of pre-treatment with forskolin (FOR; 30 μ M, 5min) on basal levels of $[Ca^{2+}]_i$ and on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (THR; 1U ml⁻¹) in bovine pulmonary artery endothelial cells, in the presence of 1.8mM extracellular calcium. In the histogram, basal and thrombin-induced elevations of $[Ca^{2+}]_i$ in control cells (open columns) and forskolin-treated cells (hatched columns) are presented as the mean of 9-22 observations and vertical bars indicate the s.e. mean.

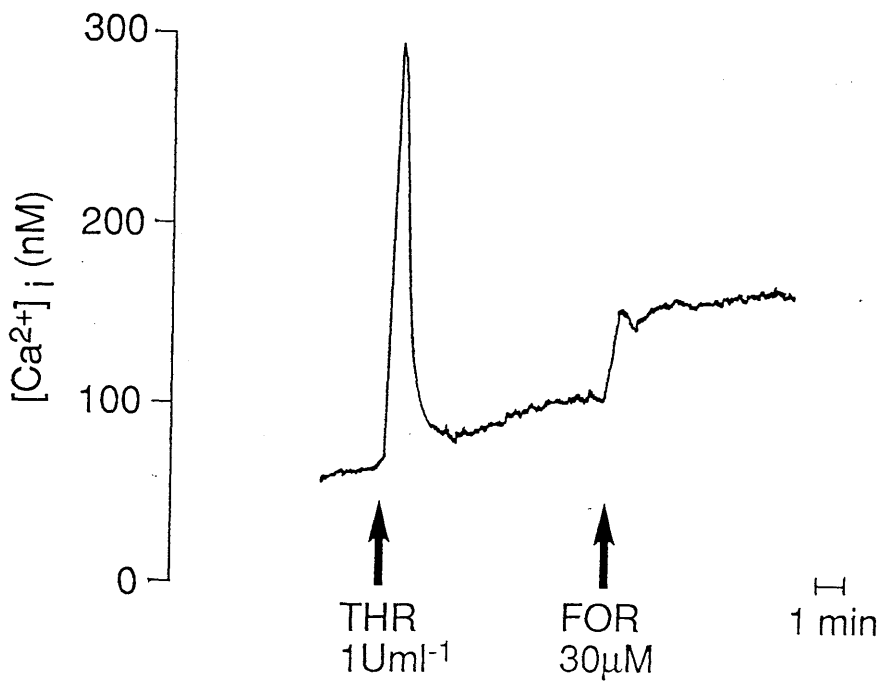


Figure 5.11 Individual trace illustrating the effects of forskolin (FOR; 30µM) on the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (THR; 1U ml⁻¹) in bovine pulmonary artery endothelial cells, in the presence of 1.8mM extracellular calcium. The trace shown is representative of 6 individual experiments.

5.5.2 Effects of atriopeptin II on thrombin-induced elevations of $[Ca^{2+}]_i$ in BPAEC

In the presence of 1.8mM extracellular calcium, pre-treatment with atriopeptin II (100nM, 5min) had no effect on basal levels of $[Ca^{2+}]_i$ or the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (1U ml⁻¹) (Figure 5.12). Addition of atriopeptin II (100nM) during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (1U ml⁻¹) resulted in a small elevation of $[Ca^{2+}]_i$ of 10-15nM (n=5; Figure 5.13).

5.5.3 Effects of 8 bromo cyclic GMP on thrombin-induced elevations of $[Ca^{2+}]_i$ in BPAEC

In the presence of 1.8mM extracellular calcium, pre-treatment with 8 bromo cyclic GMP (30μM, 5min) had no effect on basal levels of $[Ca^{2+}]_i$ or on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (Figure 5.14). Addition of 8 bromo cyclic GMP (30μM) during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (1U ml⁻¹) resulted in a small elevation of $[Ca^{2+}]_i$ of 10-15nM (n=3; Figure 5.13).

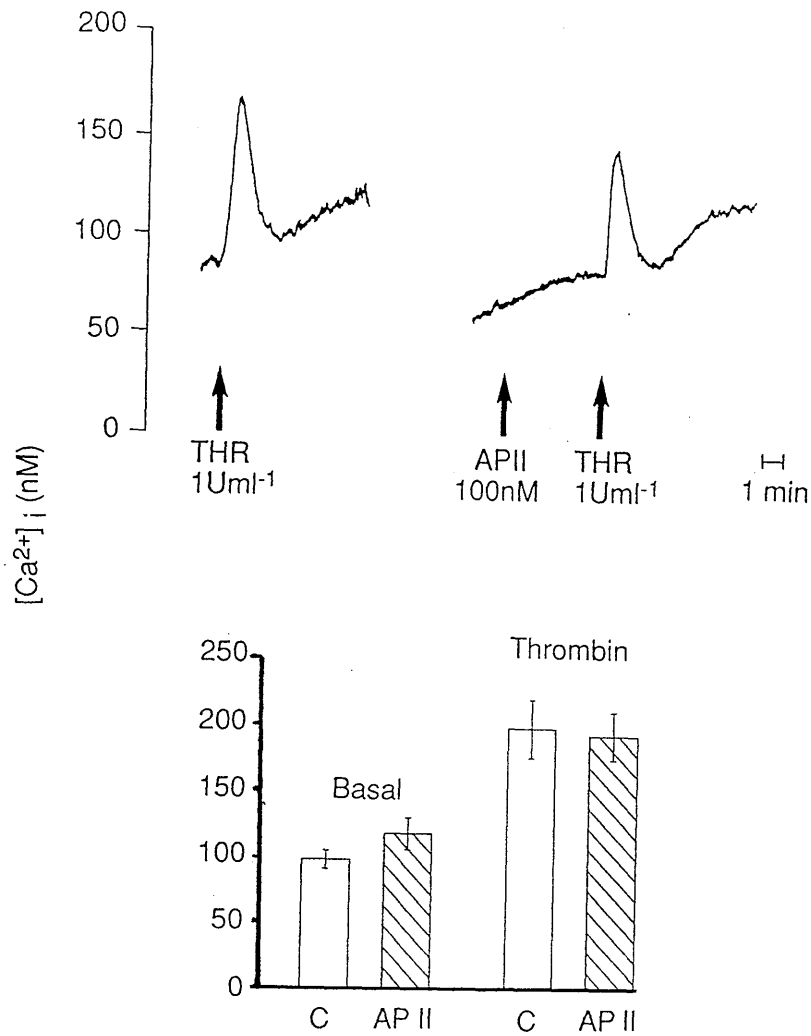


Figure 5.12 Individual traces (a) and a histogram (b) illustrating effects of pre-treatment with atriopeptin II (APII; 100nM, 5min) on basal levels of $[Ca^{2+}]_i$ and on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (THR; 1U ml⁻¹) in bovine pulmonary artery endothelial cells, in the presence of 1.8mM extracellular calcium. In the histogram, basal and thrombin-induced elevations of $[Ca^{2+}]_i$ in control cells (open columns) and atriopeptin II-treated cells (hatched columns) are presented as the mean of 6-13 observations and vertical bars indicate the s.e. mean.

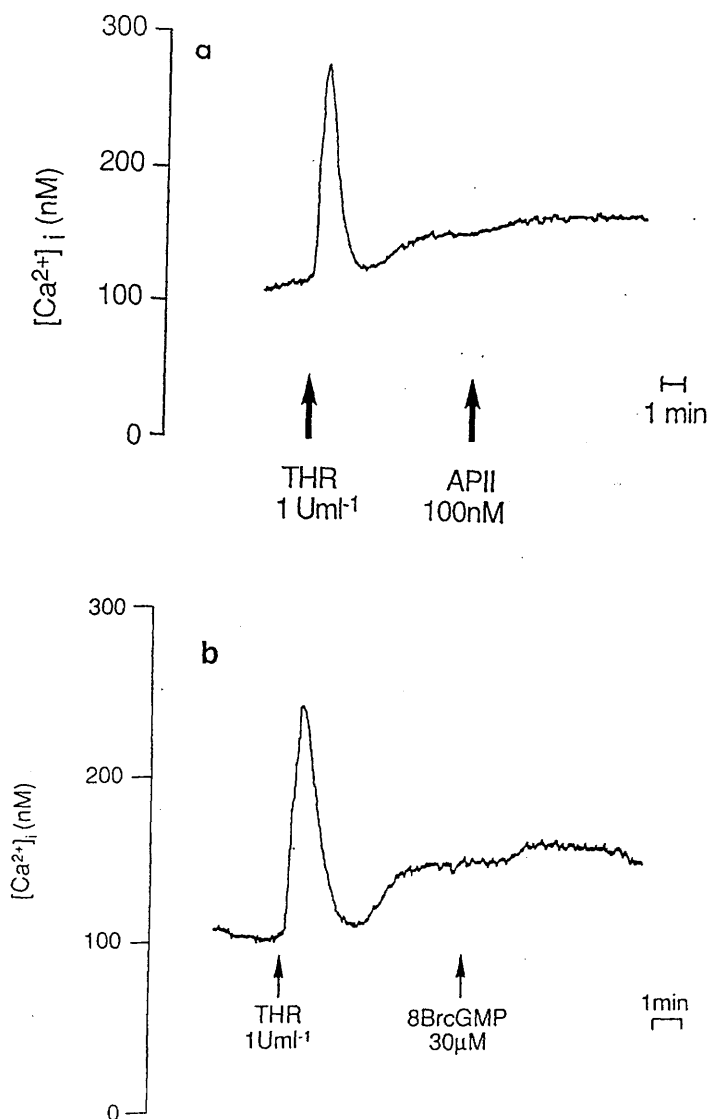


Figure 5.13 Individual traces illustrating the effects of atriopeptin II (a; APII; 100nM) and 8 bromo cyclic GMP (b; 8BrcGMP; 30μM) on the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (THR; 1U ml⁻¹) in bovine pulmonary artery endothelial cells, in the presence of 1.8mM extracellular calcium. Traces (a) and (b) are representative of 5 and 3 individual experiments, respectively.

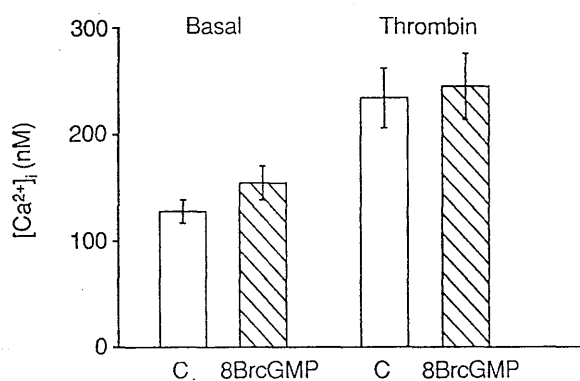
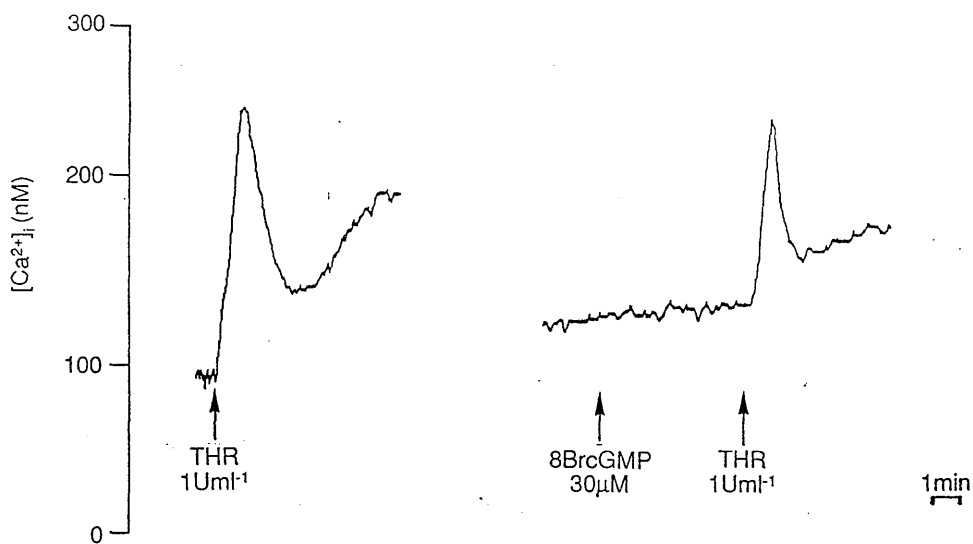


Figure 5.14 Individual traces (a) and histogram (b) illustrating the effects of pre-treatment with 8 bromo cyclic GMP (8BrcGMP; $30\mu\text{M}$, 5min) on basal levels of $[\text{Ca}^{2+}]_i$ and the magnitude of the initial transient elevation of $[\text{Ca}^{2+}]_i$ induced by thrombin (THR; 1U ml^{-1}) in bovine pulmonary artery endothelial cells, in the presence of 1.8mM extracellular calcium. In the histogram, basal and thrombin-induced elevations of $[\text{Ca}^{2+}]_i$ in control cells (open columns) and 8BrcGMP-treated cells (hatched columns) are presented as the mean of 4-10 observations and vertical bars indicate the s.e. mean.

DISCUSSION

Regulation of $[Ca^{2+}]_i$ in bovine aortic endothelial cells

6.1 Role of intracellular and extracellular pools

In this study, cytosolic calcium ($[Ca^{2+}]_i$) was measured using the fluorescent dye, fura-2 (Grynkiewicz et al., 1985). The advantages of this dye over its predecessor, quin 2, have been discussed in the methods and include greater resolution at higher calcium concentrations, higher quantum yields and the ability to measure $[Ca^{2+}]_i$ in ratio mode. With fura-2, ratio mode involves the measurement of fluorescence, following excitation at two separate wavelengths (340nm and 380nm). The ratio of these two fluorescence values provides a measure of the intracellular calcium concentration which is independent of changes in dye concentrations or instrumental variability. Fura-2 studies were undertaken using endothelial cells grown on glass coverslips. This is preferable to using cell suspensions as endothelial cells are anchorage-dependent and, therefore, this system more closely resembles the in situ situation. Furthermore, cells in suspension are more susceptible to shear stress, which may lead to partial activation of the cells.

In this study, basal levels of $[Ca^{2+}]_i$ in bovine aortic endothelial cells were approximately 100nM. This is similar to the values of 60-100nM found by other workers in endothelial cell studies (Lückhoff & Büsse, 1986; Hallam &

Pearson, 1986; Hallam et al., 1988a, b). Bradykinin, thrombin and adenosine trisphosphate (ATP) each promote the release of prostacyclin and endothelium-derived relaxing factor (EDRF) from the endothelium (Weksler et al., 1978; Pearson et al., 1983; Furchgott, 1984; White & Martin, 1989), and were each found to induce biphasic elevations of $[Ca^{2+}]_i$ in bovine aortic endothelial cells, in this study. These biphasic elevations of $[Ca^{2+}]_i$ consisted of a large, transient component, which peaked within 30 seconds, followed by a lower, well-sustained component (the plateau phase of the increase in $[Ca^{2+}]_i$), which was maintained for at least 10-15 minutes. These findings are in agreement with previous observations made in endothelial cells isolated from bovine aorta (Colden-Stanfield et al., 1987; Lückhoff et al., 1988b), human umbilical vein (Rotrosen & Gallin, 1986; Hallam et al., 1988a, b; Brock & Capasso, 1988), bovine pulmonary artery (Ryan et al., 1988) and pig aorta (Hallam & Pearson, 1986).

Endothelial shape change and release of EDRF are induced by platelet activating factor (PAF) and histamine, respectively (Grigorian & Ryan, 1987; Furchgott, 1984) and these agents have been previously shown to induce calcium mobilisation in endothelial cells derived from bovine pulmonary artery and bovine aorta, respectively (Brock & Gimbrone, 1986; Marsden et al., 1990). In this study, these agents had no effect on $[Ca^{2+}]_i$ in bovine aortic endothelial cells. However, in the earlier studies, the elevations of $[Ca^{2+}]_i$ induced by these agents were small in

magnitude and their reproducibility may depend upon variable factors, such as the method of cell isolation or culture conditions. Venous endothelium appears to be much more responsive to the actions of histamine, which induces large elevations of $[Ca^{2+}]_i$ in human umbilical vein endothelial cells (Rotrosen & Gallin, 1986; Hallam et al., 1988b).

Although lipopolysaccharide has been previously shown to induce release of EDRF (Salvemini et al., 1989), we found no evidence that this agent induces calcium mobilisation in bovine aortic endothelial cells and, therefore, it may promote EDRF release by a calcium-independent mechanism.

Ionomycin, a calcium ionophore (Liu & Hermann, 1978) was used, during experimental calibrations to promote the entry of both calcium and manganese. Ionomycin was found to elevate $[Ca^{2+}]_i$ in bovine aortic endothelial cells and, at higher concentrations of this agent, $[Ca^{2+}]_i$ was elevated to a level which saturated the intracellular fura-2. Although the major mechanism of action of ionomycin is likely to involve the transfer of calcium ions across the plasma membrane into the cytosol, the ability of this agent to induce a transient elevation of $[Ca^{2+}]_i$, in the absence of extracellular calcium, suggests that it acts on an intracellular calcium pool, which is presumably the endoplasmic reticulum.

The plateau phase of the increases in $[Ca^{2+}]_i$ induced by bradykinin and ATP, the two agonists which induced the greatest elevations of $[Ca^{2+}]_i$, were abolished when bovine aortic endothelial cells were placed in nominally calcium-free solution containing 0.5mM EGTA, giving an extracellular calcium concentration of approximately 10nM. This observation has been made in all endothelial cell types studied (Hallam & Pearson, 1986; Rotrosen & Gallin, 1986; Colden-Stanfield et al., 1987). In the presence of 1.8mM extracellular calcium, the plateau phase of the increase in $[Ca^{2+}]_i$ was rapidly terminated by the action of nickel, which has previously been shown to block calcium influx into endothelial cells (Hallam et al., 1988b). Furthermore, this component was not obtained if cells had been pre-treated with nickel. These observations suggest that the plateau phase of the increase in $[Ca^{2+}]_i$ is dependent on calcium influx, and this is supported by studies on $^{45}Ca^{2+}$ flux (Bussolino et al., 1985), patch clamp analysis (Johns et al., 1987) and the finding that agonists can promote entry of another divalent cation, manganese, into endothelial cells (Hallam et al., 1988b).

In this study, the plateau phase of the increase in $[Ca^{2+}]_i$ was found to be sustained and substantially elevated above basal $[Ca^{2+}]_i$ for several minutes. However, single endothelial cells rarely display this behaviour and, usually, repetitive oscillations in $[Ca^{2+}]_i$ are observed (Jacob et al., 1988). Recent studies undertaken by Carter et al. (1990) suggest that elevations of $[Ca^{2+}]_i$ obtained

in single endothelial cells may be heterogenous in nature, and this is illustrated in Figure 6.1. Hence, when recording from a monolayer population of cells, the fura-2 fluorescent signal is likely to represent the mean signal from thousands of cells and, therefore, if these cells are oscillating out of synchrony, then the resulting calcium signal will be displayed as a sustained elevation of $[Ca^{2+}]_i$. In this study, oscillations in $[Ca^{2+}]_i$ were observed in a small number of experiments with bradykinin (2 from approximately 400 experiments), but only at the lowest concentration examined (0.3nM). Oscillations in $[Ca^{2+}]_i$, following fluorescence measurement from a large population of endothelial cells, have also been observed in other studies (Sage et al., 1989; Neylon & Irvine, 1990). These calcium oscillations are likely to result from the cycling of calcium between the cytosol and intracellular calcium stores and are sustained by calcium influx.

Calcium influx is unlikely to occur via voltage-operated channels (VOCs) in endothelial cells, as electrophysiological studies have failed to show the existence of these channels (Colden-Stanfield et al., 1987). In this study, depolarisation with potassium chloride had no effect on basal $[Ca^{2+}]_i$ in bovine aortic endothelial cells, as observed previously in pig aortic and bovine pulmonary artery endothelial cells (Hallam & Pearson, 1986; Colden-Stanfield et al., 1987). The inability of organic calcium channel antagonists to block agonist-induced elevations of $[Ca^{2+}]_i$ (Colden-Stanfield et al., 1987), or

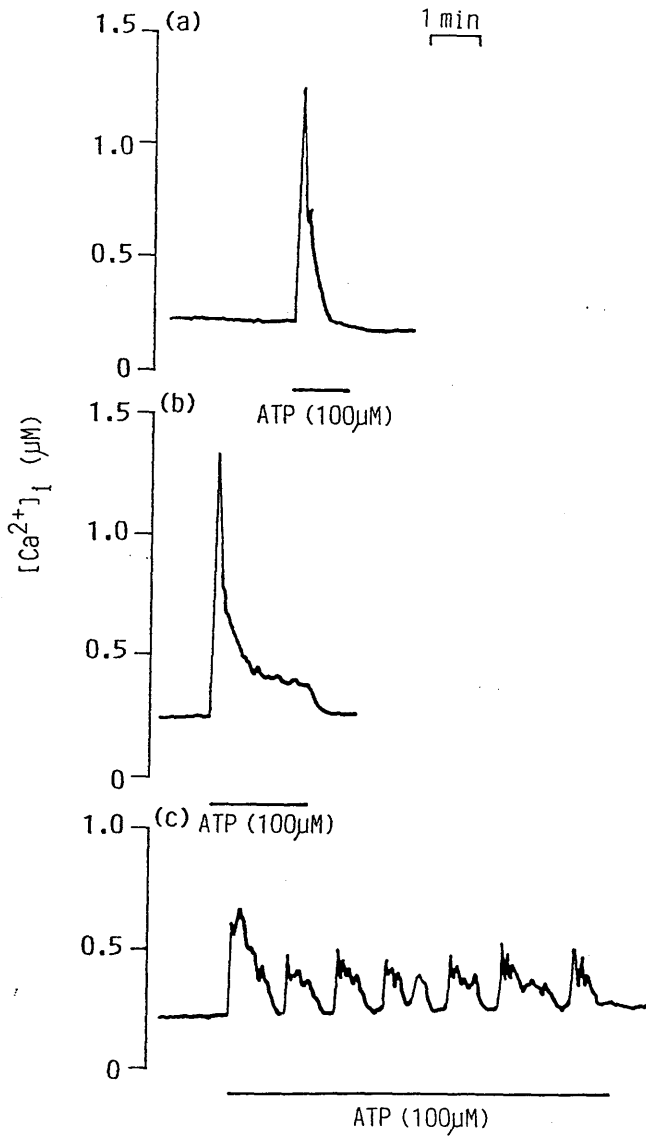


Figure 6.1 Individual traces representing possible changes in $[Ca^{2+}]_i$ observed when recording from single endothelial cells. (a) a single, transient elevation of $[Ca^{2+}]_i$, (b) an initial transient elevation of $[Ca^{2+}]_i$, followed by a sustained plateau phase and (c) an initial transient elevation of $[Ca^{2+}]_i$, followed by oscillations in $[Ca^{2+}]_i$. Reproduced from Carter et al. (1990).

endothelium-dependent relaxations (Jayakody et al., 1987), suggests that calcium influx occurs via receptor-operated channels (ROCs). However, these ROCs display some membrane potential sensitivity as, in this study, it was found that the addition of potassium chloride during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by bradykinin, thrombin and ATP resulted in a fall in $[Ca^{2+}]_i$, which was not well sustained. Similar inhibition of calcium influx by membrane depolarisation has been observed in other studies (Laskey et al., 1990; Lückhoff & Busse, 1990) and may result from a reduction in the electrochemical gradient for calcium entry through the ROCs. In this study, potassium chloride appeared to attenuate calcium influx to a lesser extent than that observed in similar studies (Laskey et al., 1990; Lückhoff & Busse, 1990). This apparent anomaly may result from the lower concentrations of potassium chloride used in this study or, alternatively, the use of a slightly hypertonic solution, in contrast to the isotonic solutions utilised in other studies. The ability of membrane depolarisation to reduce calcium influx in the endothelium and, subsequently, to reduce EDRF release (Lückhoff & Busse, 1990), may explain, in part, why acetylcholine-induced EDRF release relaxes noradrenaline-induced contractions of vascular smooth muscle much more easily than potassium chloride-induced contractions (Collins et al., 1988).

The magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by bradykinin was also attenuated in the presence

of high concentrations of potassium chloride, although this concentration had no effect on basal $[Ca^{2+}]_i$. The mechanism of this inhibition is not clear, although it may result from the hyperosmolarity of the final potassium-containing solution.

The mechanism of calcium entry through ROCs, in the endothelium, is not known. It may involve direct coupling between the receptor and the ion channel, as observed in response to ATP in vascular smooth muscle (Benham & Tsien, 1987). Alternatively, inositol (1,3,4,5) tetrakisphosphate has been implicated in the control of calcium influx (Irvine & Moor, 1986, 1987), although this tetrakisphosphate isomer may not be present in the endothelium (Pollock et al., 1988). Another possibility is that calcium entry may occur via a mechanism related to the capacitative model (Putney, 1990), which proposes that calcium influx is promoted subsequent to the inositol (1,4,5) trisphosphate-mediated discharge of calcium stores. This model is operational in parotid acinar cells, where the inositol phosphate-mediated discharge of intracellular calcium stores results in calcium influx (Bird et al., 1991).

As we wished to determine whether the intracellular calcium stores were in equilibrium with extracellular calcium, we examined the effects of removal of extracellular calcium. When bovine aortic endothelial cells were bathed in nominally calcium-free solution

containing 0.5mM EGTA, the magnitude of the initial transient elevations of $[Ca^{2+}]_i$ was significantly reduced with all concentrations of bradykinin and ATP examined. This observation is consistent with the findings of Colden-Stanfield et al. (1987) and Schilling et al. (1988). In a separate study, in which no statistical validation was presented (Lückhoff et al., 1988b), no reduction in the magnitude of this component was observed in bovine aortic endothelial cells. Under similar, nominally calcium-free conditions, variable reductions in the magnitude of the initial transient component have been observed in human umbilical vein endothelial cells, in response to histamine or thrombin (Hallam et al., 1988a; Brock & Capasso, 1988). In contrast, in a study in which the older, less sensitive probe, quin 2, was used, no reduction in the magnitude of the transient elevation of $[Ca^{2+}]_i$ was reported (Rotrosen & Gallin, 1986). In pig aortic endothelial cells, the initial transient component induced by ATP was also reduced slightly in calcium-free conditions (Hallam & Pearson, 1986). In conclusion, in statistically-validated studies using the more sensitive probe, fura-2, there appears to be general agreement that the magnitude of the initial agonist-induced transient elevations of $[Ca^{2+}]_i$ is reduced in calcium-free solutions.

From our studies performed in nominally calcium-free solutions, it was not possible to determine whether the reduced magnitude of the bradykinin-induced initial transient elevation of $[Ca^{2+}]_i$ was due to an impaired

ability to release intracellular calcium, to the depletion of an intracellular store or to loss of a transient calcium influx component. Discharge of calcium from intracellular stores is mediated via $\text{Ins}(1,4,5)\text{P}_3$ (Streb et al., 1983). However, as bradykinin-induced hydrolysis of phosphoinositides is not inhibited when bovine aortic endothelial cells are placed in calcium-free solution, it is unlikely that reduced production of $\text{Ins}(1,4,5)\text{P}_3$ accounts for the reduction in the initial transient elevation of $[\text{Ca}^{2+}]_i$. Loss of a calcium influx component is also unlikely since, in the presence of extracellular calcium, treatment with nickel, which blocks calcium influx (Hallam et al., 1988b), had no effect on the magnitude on the bradykinin-induced initial transient elevation of $[\text{Ca}^{2+}]_i$ in this study, although the plateau phase was abolished. Lanthanum, another inhibitor of calcium influx, was similarly found to have little effect on the magnitude of the initial transient elevation of $[\text{Ca}^{2+}]_i$, despite blocking the sustained component (Colden-Stanfield et al., 1987). Our novel observation that the ability of caffeine to release intracellular calcium (Weber & Herz, 1968), and elevate $[\text{Ca}^{2+}]_i$ in bovine aortic endothelial cells, was blocked in the absence of extracellular calcium, is consistent with the depletion of intracellular stores. The small magnitude of the elevation of $[\text{Ca}^{2+}]_i$ induced, however, shows that the caffeine-sensitive calcium pool is a small proportion of the total intracellular content. It is likely, therefore, that the bradykinin-induced initial transient elevation of $[\text{Ca}^{2+}]_i$ is completely dependent upon

release of calcium from an intracellular store and that exposure to calcium-free solution leads to depletion of this store.

The reduction of this initial, transient elevation of $[Ca^{2+}]_i$, in the absence of extracellular calcium, has been correlated with functional changes. For example, release of prostacyclin from the endothelium is closely correlated to the initial transient elevation of $[Ca^{2+}]_i$ (Hallam et al., 1988a). In pig aortic endothelial cells, removal of extracellular calcium has little effect on prostacyclin release or on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ (Hallam & Pearson, 1986; White & Martin, 1989). In contrast, in this study, in bovine aortic endothelial cells, removal of extracellular calcium significantly reduced the initial calcium transient and this corresponds to a significant reduction in prostacyclin production in these cells, as observed by Lückhoff and co-workers (1988a).

In a separate series of experiments, an attempt was made to examine the kinetics of the loss of the bradykinin-induced initial transient elevation of $[Ca^{2+}]_i$ in calcium-free solution, in bovine aortic endothelial cells. In these experiments, cells were bathed in 1mM extracellular calcium and then exposed to EGTA, a calcium chelator, for different times and at different concentrations. Exposure to EGTA (2mM), for only 1 minute, reduced the magnitude of the bradykinin-induced transient elevation of $[Ca^{2+}]_i$ by 50%.

However, increasing the concentration of EGTA or the time of exposure resulted in no further reduction in the bradykinin-induced initial transient elevation of $[Ca^{2+}]_i$. These novel observations suggest the possible existence of two intracellular calcium pools; one which is rapidly depleted in the presence of low extracellular calcium and a second which is resistant even to prolonged removal of extracellular calcium. The rapidly depleted store appears to contribute more to responses obtained to sub-maximal stimuli since, in the presence of EGTA, the initial transient elevation of $[Ca^{2+}]_i$ is blocked to a greater degree when the concentration of bradykinin is low. Freay et al. (1989) have described the existence of two, non-mitochondrial calcium pools in bovine pulmonary artery endothelial cells, only one of which is sensitive to the actions of $Ins(1,4,5)P_3$. Ghosh et al. (1989) have described the presence of separate guanosine triphosphate(GTP)-sensitive and $Ins(1,4,5)P_3$ -sensitive pools in smooth muscle cells. Whether these calcium pools are equivalent to the two pools suggested by our findings remains to be determined, although Freay et al. (1989) found that GTP does not modulate $Ins(1,4,5)P_3$ -induced release of calcium from intracellular stores in bovine pulmonary artery endothelial cells.

When bovine aortic endothelial cells were bathed in nominally calcium-free solution in the presence of 0.5mM EGTA, the bradykinin - or ATP-induced transient elevation of $[Ca^{2+}]_i$ was complete within 90s. In a separate series

of experiments, we found that subsequent re-addition of extracellular calcium, to achieve a free concentration of around 1.8mM, resulted in a biphasic elevation of $[Ca^{2+}]_i$, consisting of a large, initial transient component, followed by a smaller, well-maintained component. It is likely that the latter is analogous to the plateau phase of the increase in $[Ca^{2+}]_i$ induced by bradykinin or ATP in the presence of extracellular calcium. However, the nature of the initial transient component is less clear, and may derive from calcium influx, further intracellular release of calcium or calcium-induced calcium release. It was certainly agonist-dependent as it was not observed when calcium was re-added to cells bathed in calcium-free medium in the absence of agonist. Procaine has been shown to abolish calcium-induced calcium release (Weber & Herz, 1968; Saida & van Breemen, 1984). However, in this study, the initial component appeared largely unchanged in the presence of procaine, although it was impossible to precisely measure $[Ca^{2+}]_i$ in the presence of procaine as this compound absorbs fura-2 fluorescence. It is, therefore, unlikely that this transient component results from calcium-induced calcium release and it may simply derive from the reloading and subsequent discharge of intracellular calcium stores, upon re-addition of extracellular calcium.

The ability of TMB-8, a putative inhibitor of intracellular calcium release (Malagodi & Chiou, 1974), to inhibit

agonist-induced prostacyclin production by endothelial cells (Seid et al., 1983; Lückhoff et al., 1988a; White & Martin, 1989) appears consistent with the belief that prostacyclin production is more dependent upon release of calcium from intracellular stores than from calcium influx (Seid et al., 1983; Lückhoff et al., 1988a, Hallam et al., 1988a; White & Martin, 1989). However, in this study, treatment with TMB-8 for up to 15 minutes had no effect on the bradykinin-induced transient elevation of $[Ca^{2+}]_i$. This indicates that TMB-8 is unable to inhibit release of calcium from intracellular stores in bovine aortic endothelial cells. This finding is supported by the observation that TMB-8 does not inhibit $Ins(1,4,5)P_3$ -induced calcium release in NG108-15 neuroblastoma x glioma cells (Campbell et al., 1990). TMB-8 did induce a small elevation of $[Ca^{2+}]_i$ by itself, which was dependent upon the presence of extracellular calcium, although the precise mechanism by which it produces this is not known. It, therefore, appears unlikely that TMB-8 inhibits the release of prostacyclin by inhibiting release of calcium from intracellular stores.

6.2 Protein kinase C and $[Ca^{2+}]_i$

As described in section 6.1., agonist-induced elevations of $[Ca^{2+}]_i$ in bovine aortic endothelial cells consist of an initial transient component, which is largely due to the release of calcium from intracellular stores, and a plateau

phase, which is due to calcium influx from the extracellular space. The tumour-promoting phorbol esters are known activators of protein kinase C (Castagna et al., 1982) and are useful tools for activating this pathway in cells. Pre-treatment of bovine aortic endothelial cells with phorbol myristate acetate (PMA), an active phorbol ester, had no effect on basal $[Ca^{2+}]_i$ but reduced the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by maximal concentrations of thrombin and low concentrations of bradykinin or ATP. In contrast, the initial transient elevation of $[Ca^{2+}]_i$ induced by maximal and near maximal concentrations of bradykinin and ATP were unaffected. These findings in bovine aortic endothelial cells contrast with the ability of phorbol esters to inhibit intracellular release of calcium induced by maximal concentrations of thrombin, bradykinin and ATP in human umbilical vein endothelial cells (Brock & Capasso, 1988; Carter et al., 1989), and may highlight a species or site difference. The ability of PMA to inhibit this initial transient elevation of $[Ca^{2+}]_i$ is likely to result from its ability to activate protein kinase C. In support of this, staurosporine, an inhibitor of protein kinase C (Tamaoki et al., 1986; Davis et al., 1989), blocked the inhibitory effect of PMA and, secondly, the inactive phorbol ester, 4 α -phorbol, 12,13-didecanoate, lacked the activity of PMA.

The initial transient elevation of $[Ca^{2+}]_i$ in many cell types is likely to result from $Ins(1,4,5)P_3$ -induced release of calcium from intracellular stores (Berridge, 1984). A

number of agents, including bradykinin, ATP, histamine and thrombin, induce hydrolysis of phosphoinositides and production of $\text{Ins}(1,4,5)\text{P}_3$ in the endothelial cell (Derian & Moskowitz, 1986; Piroton et al., 1987; Pollock et al., 1988; Brock & Capasso, 1988). Therefore, the ability of phorbol esters to inhibit production of $\text{Ins}(1,4,5)\text{P}_3$ in the endothelium (Brock & Capasso, 1988; Halldorsson et al., 1988; Carter et al., 1989) is likely to account for the ability of these compounds to inhibit the initial transient elevation of $[\text{Ca}^{2+}]_i$. However, in bovine aortic endothelial cells, PMA differentially inhibits release of intracellular calcium induced by different agonists and this may reflect the extent to which receptor-mediated $\text{Ins}(1,4,5)\text{P}_3$ production may be inhibited by protein kinase C. Alternatively, at low agonist concentrations, where $\text{Ins}(1,4,5)\text{P}_3$ generation may be limited, other mechanisms may contribute to the inhibitory actions of PMA: for example, activation of protein kinase C may promote breakdown of $\text{Ins}(1,4,5)\text{P}_3$ (Connolly et al., 1986) or raise the threshold for activation of the $\text{Ins}(1,4,5)\text{P}_3$ receptor (Willems et al., 1989).

As described in section 6.1, the sustained plateau phase of the agonist-induced increase in $[\text{Ca}^{2+}]_i$ results from calcium influx through receptor-operated channels. In this study, it was found that PMA induces a fall in $[\text{Ca}^{2+}]_i$ when added during the plateau phase of the increase in $[\text{Ca}^{2+}]_i$ induced by maximal or submaximal concentrations of thrombin, bradykinin or ATP. Hence, this component of the

biphasic response is much more sensitive to the actions of PMA than is the large, transient intracellular release component. This is in contrast to platelets and submandibular duct cells, where the calcium influx component is less sensitive to the actions of the phorbol esters (Valone et al., 1987; He et al., 1988). EDRF production is calcium-dependent (Singer & Peach, 1982; Long & Stone, 1985) and this is likely to result from the calcium-dependence of nitric oxide synthase (Meyer et al., 1989). As nitrovasodilator-induced relaxations are unaffected by phorbol esters (Lewis & Henderson, 1987), it is likely that the phorbol esters inhibit the production of EDRF. Furthermore, the inability of phorbol esters to inhibit endothelium-dependent relaxations induced by the calcium ionophore, A23187 (Weinheimer et al., 1986; Lewis & Henderson, 1987; Cherry & Gillis, 1988), suggests that these agents inhibit EDRF release via the inhibition of receptor-mediated calcium mobilisation. However, receptor-mediated agonists are not all equally sensitive to inhibition: for example, Smith and Lang (1990) have shown that substance P - and ATP-induced production of EDRF in pig aortic endothelial cells, is inhibited by phorbol esters, although that induced by bradykinin is unaffected. This differential sensitivity may be explained by our finding that bradykinin-induced calcium mobilisation, both from intracellular and extracellular pools, in bovine aortic endothelial cells, is less sensitive to the inhibitory actions of phorbol esters than that induced by other agonists.

The mechanism of action of PMA was examined in greater detail to determine whether or not it inhibits the agonist-induced plateau phase of $[Ca^{2+}]_i$ via promotion of calcium efflux, inhibition of calcium influx, or a combination of these mechanisms. By making use of the ability of manganese to quench fura-2 fluorescence, Hallam et al. (1988b) showed that agonists promote entry of manganese into the endothelial cell by a similar route to that used by calcium. Hence, agonist-induced quenching of cytosolic fura-2 by manganese can be taken as indirect evidence of the opening of channels that permit calcium influx. In this study, thrombin increased the rate at which manganese quenched fura-2 fluorescence, indicating that it promoted calcium influx. PMA inhibited the ability of thrombin to increase the quenching of fura-2 fluorescence, but the inactive phorbol ester, 4 α -PDD did not. It is likely, therefore, that activation of protein kinase C by PMA inhibits calcium influx.

In a number of cell types, for example, neutrophils, platelets and smooth muscle, phorbol esters activate the plasma membrane Ca^{2+} ATPase and, therefore, promote calcium efflux (Lagast et al., 1984; Rink & Sage, 1987; Rashatwar et al., 1987). Experimentally, this action may be demonstrated by the ability of phorbol esters to reduce the sustained elevation of $[Ca^{2+}]_i$ induced by a low concentration of a calcium ionophore, as observed in

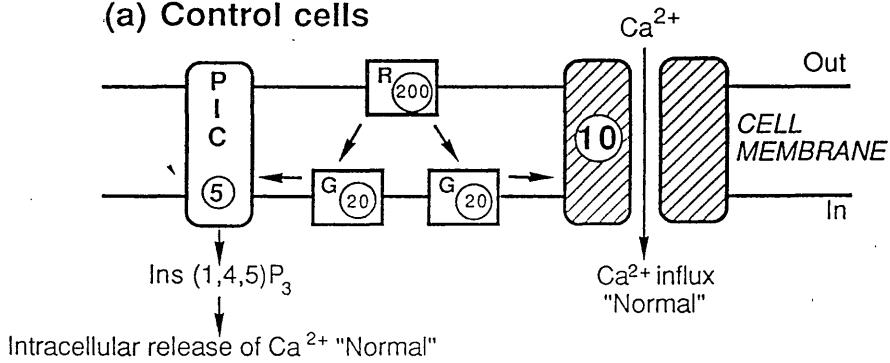
neutrophils (MacCarthy et al., 1989). However, in this study, PMA had no effect on ionomycin-induced elevations of $[Ca^{2+}]_i$, suggesting that activation of protein kinase C does not stimulate calcium extrusion in bovine aortic endothelial cells.

Although the site of action of protein kinase C is not known, possible substrates for phosphorylation include membrane receptors, G-proteins, phosphoinositidase C or calcium channels. A number of studies have demonstrated the inhibition of G-protein function by protein kinase C (Smith et al., 1987; Orellana et al., 1987; Bochakov et al., 1990), which may subsequently result in reduced phosphoinositide hydrolysis and calcium mobilisation. It is possible that membrane receptors may be coupled to calcium mobilisation via distinct G proteins which differ in their susceptibility to inhibition by protein kinase C. This is supported by the work of Voyno - Yassenetskaya et al. (1989b) who showed the ability of pertussis toxin to inhibit histamine, but not bradykinin-induced phosphoinositide hydrolysis in human umbilical vein endothelial cells. This may provide an explanation for the differential agonist sensitivity to the phorbol esters, demonstrated in this study and that of Smith and Lang (1990). The finding of this study that agonist-induced influx of calcium is more sensitive to inhibition by PMA, than is intracellular calcium release, requires an explanation. Theoretically, an alteration in the

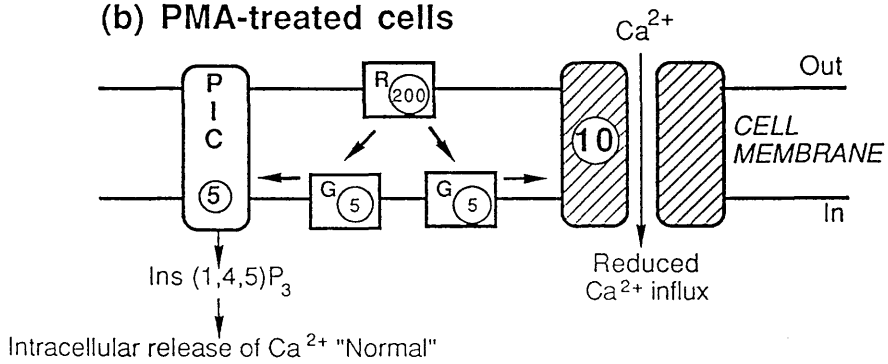
proportion of functional G proteins, relative to the appropriate cellular effector, could underly this differential sensitivity. According to this scheme, it is possible that intracellular release of calcium (via $\text{Ins}(1,4,5)\text{P}_3$) and calcium influx are both functionally coupled to G proteins. In untreated cells, functional G proteins may be greatly in excess of both the cellular effector systems (Figure 6.2a). However, in PMA-treated cells, the number of functional G proteins may fall to levels which are insufficient to promote maximal activation of the calcium influx channel, but which still allow maximal activation of phosphoinositidase C and, therefore, maximal release of calcium from intracellular stores via $\text{Ins}(1,4,5)\text{P}_3$ (Figure 6.2b). However, if the number of functional G-proteins falls further, then intracellular release of calcium will also be inhibited (Figure 6.2c).

The finding of this study that staurosporine increases the magnitude of the plateau phase of the increase in $[\text{Ca}^{2+}]_i$ induced by bradykinin and thrombin is likely to reflect the ability of these agonists to activate protein kinase C themselves, and so inhibit their own actions by a negative feedback loop. These agonists may activate protein kinase C by inducing hydrolysis of phosphoinositides (Derian & Moskowitz, 1986; Halldorsson et al., 1988) or, alternatively, phosphatidylcholine (Michaelis & Martin, 1988). In contrast, staurosporine induced a fall in the plateau phase of the increase in $[\text{Ca}^{2+}]_i$ induced by ATP. This may result from the ability of staurosporine to

(a) Control cells



(b) PMA-treated cells



c) Further treatment with PMA

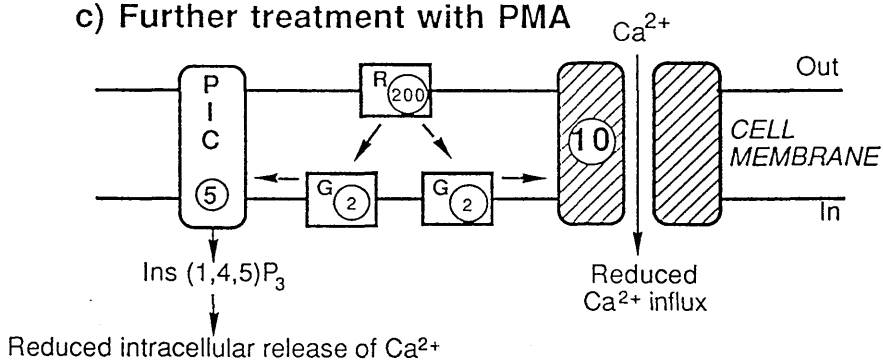


Figure 6.2 A diagrammatic representation of a theory which may explain the changes in cellular messenger systems which underlie the greater sensitivity of intracellular release of calcium, in comparison to calcium influx, to the actions of the phorbol esters. In this scheme, the processes controlling intracellular release of calcium and calcium influx are coupled to membrane-bound receptors via regulatory G-proteins (Figure 6.2a). The numbers associated with each receptor, G-protein and effector indicate the proportional number of each protein associated with the system. Treatment with phorbol esters may lower the number of functional G proteins in the cell (Figure 6.2b). However, this number of G-proteins may still be sufficient to fully activate phosphoinositidase C (PIC) and, therefore, maximally activate intracellular release of calcium. However, following further treatment with phorbol esters, if the number of functional G-proteins falls below the level required for maximal activation of PIC, then intracellular release of calcium will also be inhibited (Figure 6.2c). (As discussed with Dr. Andrew Newby.)

interact with the ATP binding sites in proteins (Davis et al., 1989).

6.3 Cyclic AMP and $[Ca^{2+}]_i$

Little is known about how cyclic AMP regulates endothelial cell function. Initial studies suggested that elevation of cyclic AMP content may inhibit prostacyclin production by a negative feedback mechanism (Adams Brotherton & Hoak, 1982), although this was later ascribed to the cyclic AMP-independent, inhibitory actions of the phosphodiesterase inhibitor, 3-isobutyl-1-methyl xanthine which was used in these studies (Adams Brotherton et al., 1982).

Elevation of cyclic AMP via prostacyclin, forskolin, calcitonin gene-related peptide and β -adrenoceptor agonists has been also proposed to augment EDRF release (Shimokawa et al., 1988; Gray & Marshall, 1991a, b; Gardiner et al., 1991). This may be explained by our observation that both forskolin and isoprenaline induced biphasic elevations of $[Ca^{2+}]_i$ when added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin, bradykinin and ATP, as EDRF production is calcium-dependent (Singer & Peach, 1982; Long & Stone, 1985). In contrast to the afore-mentioned studies, Kuhn et al. (1991) found that elevation of cyclic AMP had no effect on bradykinin-induced EDRF release from bovine aortic endothelial cells. However, in this latter study, the concentration of bradykinin used was maximal and, therefore, it is possible that the elevation of

$[Ca^{2+}]_i$ was sufficient to fully activate nitric oxide synthase, making an additional augmentation of enzyme activity, in response to a further increase in $[Ca^{2+}]_i$, impossible.

The biphasic elevations of $[Ca^{2+}]_i$ observed, in this study, when forskolin or isoprenaline was added during the agonist-induced elevation of $[Ca^{2+}]_i$, in bovine aortic endothelial cells, consisted of a large, initial transient elevation, followed by a lower, more sustained plateau phase. As neither forskolin nor isoprenaline had any effect on basal $[Ca^{2+}]_i$, it is likely that their actions result only from the facilitation of agonist-induced calcium mobilisation. This is in contrast to the ability of cyclic AMP to independently release an intracellular calcium pool in T lymphocytes (Kelley et al., 1990). The initial transient and sustained phases of the increases in $[Ca^{2+}]_i$, induced by forskolin and isoprenaline, are likely to result from intracellular release and calcium influx, respectively, since only the latter was abolished in the presence of the calcium entry blocker, nickel. Furthermore, although it has been proposed that staurosporine is a non-selective protein kinase inhibitor (Davis et al., 1989), it had no effect on the forskolin-induced augmentation of calcium mobilisation, suggesting that its actions in the endothelium are selective for protein kinase C.

The above explanation of the cyclic AMP-mediated enhancement of calcium mobilisation may be somewhat simplistic. As discussed in section 6.1, the agonist-induced plateau phase of the increase in $[Ca^{2+}]_i$, obtained from a population of cells, may simply result from the mean signal from thousands of cells which are undergoing calcium oscillations out of synchrony. These calcium oscillations are likely to derive from the constant cycling of calcium between intracellular pools and the cytosol and are ultimately dependent upon calcium influx. It is likely that the biphasic augmentation of $[Ca^{2+}]_i$ observed in this study, in populations of bovine aortic endothelial cells, may simply represent an enhanced frequency and amplitude of calcium oscillations at the single cell level, as observed in hepatocytes (Schöfl et al., 1991). The mechanism by which cyclic AMP enhances agonist-induced oscillations in $[Ca^{2+}]_i$ may involve facilitation of calcium influx, as observed in hepatocytes (Poggioli et al., 1986) and cardiac myocytes (Cachelin et al., 1983). An additional mechanism of action of cyclic AMP may involve modification of the properties of the intracellular calcium stores: for example, enhanced uptake into intracellular stores may occur, as observed in cardiac myocytes (Fabiato & Fabiato, 1975; Mueller & van Breemen, 1979) or in smooth muscle (Itoh et al., 1985). If this occurs in the endothelial cell, it will result in the increased cycling of calcium between $Ins(1,4,5)P_3$ -sensitive calcium stores and the cytosol.

In addition to a direct effect on cellular calcium mobilisation, it is possible that cyclic AMP could augment $[Ca^{2+}]_i$ indirectly. As discussed in section 6.2, activation of protein kinase C inhibits agonist-induced elevations of $[Ca^{2+}]_i$ in bovine aortic endothelial cells. Furthermore, elevation of cyclic AMP content inhibits the actions of protein kinase C in the endothelium (Gudgeon & Martin, 1989), at least when considering endothelial barrier function. It is unlikely that forskolin enhances calcium mobilisation in bovine aortic endothelial cells by inhibiting ongoing protein kinase C activity, as it was found that forskolin was unable to attenuate the ability of PMA to inhibit the plateau phase of the agonist-induced increase in $[Ca^{2+}]_i$.

This study has demonstrated that elevating the intracellular cyclic AMP content, during the agonist-induced plateau phase of the increase in $[Ca^{2+}]_i$, clearly augments intracellular release of calcium. However, this study using bovine aortic endothelial cells, and that of Carson et al. (1989), using human umbilical vein endothelial cells, both show that agents which elevate endothelial cyclic AMP content have no effect on the magnitude of the agonist-induced initial transient elevation of $[Ca^{2+}]_i$. The observation of an augmentation of intracellular release during the plateau phase, but not the initial transient elevation is likely to result from the small magnitude of this augmentation, relative to the

magnitude of the discharge that normally underlies the initial transient.

It is possible that the enhanced release of calcium from intracellular stores, observed during the plateau phase, may be dependent upon enhanced uptake into the agonist-sensitive pool, either from other intracellular stores or from the extracellular pool. Therefore, if cells are treated, before agonist addition, with a cyclic AMP-elevating agent, the intracellular calcium stores may often be at full capacity and, therefore, unable to be further loaded. Subsequently, upon agonist addition, the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ would be identical in both control cells and treated cells. In one study, elevating cyclic AMP content has been reported to augment slightly the ATP-induced initial transient elevation of $[Ca^{2+}]_i$ in bovine aortic endothelial cells (Brock et al., 1988). All of these findings, demonstrating either no effect on calcium mobilisation, or augmentation of calcium mobilisation, clearly conflict with those of Lückhoff et al. (1990), who reported that elevating cyclic AMP content inhibits endothelial calcium mobilisation in bovine aortic endothelial cells. Why the results of this latter group differ so markedly from all other published reports is not clear.

6.4 Cyclic GMP and $[Ca^{2+}]_i$

Endothelial cyclic GMP content is elevated by atrial natriuretic factors (Leitman & Murad, 1986; Martin et al.,

1988) or by agents which induce production of EDRF (Martin et al., 1988). It has been proposed that EDRF regulates its own production via a negative feedback mechanism involving cyclic GMP (Evans et al., 1988; Hogan et al., 1989). Cyclic GMP has also been reported to modulate other endothelial functions; for example, it inhibits production of thromboxane A_2 and endothelin (Worthington & Fuller, 1983; Saijonmaa et al., 1990; Boulanger & Lüscher, 1990). Cyclic GMP has been demonstrated to inhibit production of $\text{Ins}(1,4,5)\text{P}_3$ in pig aortic endothelial cells (Lang & Lewis, 1991a), but in this study, in bovine aortic endothelial cells, elevation of cyclic GMP content did not modulate either basal $[\text{Ca}^{2+}]_i$ or elevations of $[\text{Ca}^{2+}]_i$ induced by thrombin, bradykinin or ATP. Ryan et al. (1988) similarly demonstrated the inability of 8 bromo cyclic GMP to inhibit the histamine-induced transient elevation of $[\text{Ca}^{2+}]_i$ in human umbilical vein endothelial cells.

As bradykinin, thrombin and ATP are known to increase EDRF production (Furchgott, 1984) and, therefore, increase endothelial cyclic GMP content, the possibility was considered that endogenously generated cyclic GMP was capable of inhibiting endothelial calcium mobilisation, making further inhibition impossible. N^G -nitro-L-arginine, an inhibitor of EDRF production (Moore et al, 1990) did not, however, modulate basal $[\text{Ca}^{2+}]_i$, the magnitude of the initial transient elevation of $[\text{Ca}^{2+}]_i$ or the plateau phase of the increase in $[\text{Ca}^{2+}]_i$ induced by bradykinin, ATP or thrombin. Therefore, this data suggests that cyclic GMP

does not modulate calcium mobilisation in bovine aortic endothelial cells. In support of these findings, a functional study has demonstrated a lack of effect of cyclic GMP on EDRF release from bovine aortic endothelial cells (Kuhn et al., 1991).

It is not entirely clear why cyclic GMP has no effect on calcium mobilisation or EDRF production in bovine aortic endothelial cells, but inhibits EDRF release from rabbit aorta and $\text{Ins}(1,4,5)\text{P}_3$ production in pig aortic endothelial cells. The results of one functional study in pig aortic endothelial cells are consistent with the inability of cyclic GMP to modulate bradykinin-induced calcium mobilisation in pig aortic endothelial cells (White & Martin, 1989). Here, bradykinin-induced production of prostacyclin was unaffected following elevation of cyclic GMP content (Martin et al., 1989), despite production being dependent upon the release of calcium from intracellular stores (Seid et al., 1983; Lückhoff et al., 1988a; Hallam et al., 1988a; White & Martin, 1989). In contrast to these findings in pig aortic endothelial cells, nitric oxide inhibited prostacyclin production in bovine aortic endothelial cells by an apparently cyclic GMP-dependent mechanism (Doni et al., 1988) and this clearly contrasts with our findings demonstrating the inability of cyclic GMP to inhibit calcium mobilisation in bovine aortic endothelial cells.

These observed differences may result from differing agonist sensitivities to the actions of cyclic GMP: for example, in rabbit aorta, EDRF release induced by either substance P or muscarinic agonists is inhibited by elevating cyclic GMP content, but that induced by ATP is not. Species differences may play their part, as thrombin-induced $\text{Ins}(1,4,5)\text{P}_3$ production is inhibited by elevation of cyclic GMP content in pig aortic endothelial cells (Lang & Lewis, 1991a), while thrombin-induced calcium mobilisation in bovine aortic endothelial cells (this study) and thrombin-induced prostacyclin production in human umbilical vein endothelial cells (Adams Brotherton, 1986) were unaffected by elevation of cyclic GMP content.

High phosphodiesterase activity and extrusion of cyclic GMP may contribute to the lack of elevation of cyclic GMP content, in response to nitrovasodilators, observed in bovine aortic endothelial cells (Schini et al., 1989) and these findings may explain the lack of effect of sodium nitroprusside on $[\text{Ca}^{2+}]_i$ in this study. However, 8 bromo cyclic GMP, a stable analogue of cyclic GMP, presumably exerts a direct effect on cellular function and its actions are unlikely to be attenuated in the presence of high phosphodiesterase activity.

The lack of effect of cyclic GMP on calcium mobilisation in bovine aortic endothelial cells may result from a lack of cyclic GMP-dependent protein kinase in these cells, as proposed by Mackie et al. (1986). However, these workers

used endothelial cells which had been passaged as many as five times, in contrast to this study, where the cells were passaged only on a single occasion. It is possible that endothelial cell characteristics may be altered following repeated passaging and the changes may include loss of certain protein kinase activities. Atrial natriuretic factor (ANF) inhibits thrombin-induced increases in vascular permeability (Baron et al., 1989) and inhibits Na-K-Cl transport (O'Donnell, 1989) in bovine aortic endothelial cells. This latter function is likely to be cyclic GMP-mediated, as the effects of ANF were mimicked by 8 bromo cyclic GMP (O'Donnell, 1989). Furthermore, oxyhaemoglobin has been shown to enhance endothelin release from bovine aortic endothelial cells, suggesting that EDRF, presumably through cyclic GMP, inhibits endothelin production in these cells (Cocks et al., 1991). Although these findings provide indirect evidence for the presence of cyclic GMP-dependent protein kinase in endothelial cells, it is possible that cyclic GMP-mediated responses occur independently of protein kinase activation, as observed in the retina (Haynes et al., 1986).

6.5 G proteins and $[Ca^{2+}]_i$

Bradykinin, thrombin and ATP have each been shown to induce hydrolysis of phosphoinositides in the endothelium (Derian & Moskowitz, 1986; Jaffe et al., 1987; Pirroton et al., 1987; Lang & Lewis, 1991a). Receptor-mediated activation of the inositol lipid pathway is thought to be regulated via G proteins, some of which are inhibited by pertussis

toxin (Cockcroft & Stutchfield, 1988). As it is apparent that agonist-induced phosphoinositide hydrolysis in the endothelium is guanine nucleotide-dependent (Brock et al., 1988; Voyno-Yasenetskaya et al., 1989a), suggesting G protein involvement, sensitivity of agonist-induced calcium mobilisation to pertussis toxin was examined in bovine aortic endothelial cells. Following bradykinin treatment with pertussis toxin, thrombin and ATP-induced elevations of $[Ca^{2+}]_i$ were completely unaffected. It is unlikely that the lack of effect of pertussis toxin results from the inability of the toxin to enter the endothelial cell as pertussis toxin substrates have been found in endothelial cells isolated from pig aorta, bovine pulmonary artery and human umbilical vein (Lambert et al., 1986; Voyno-Yasenetskaya et al., 1989a, b). Our findings are consistent with the lack of effect of pertussis toxin treatment on bradykinin-induced hydrolysis of phosphoinositides in endothelial cells derived from the above three sites (Lambert et al., 1986; Voyno-Yasenetskaya et al., 1989a, b), but contrast with the pertussis toxin sensitivity of the thrombin-activated G protein in human umbilical vein endothelial cells (Lampugnani et al., 1990). A small inhibition of ATP-induced inositol phosphate production in bovine aortic endothelial cells has been previously found, following treatment with pertussis toxin (Piroton et al., 1987). It is not entirely clear why no inhibition of the ATP-induced initial transient elevation of $[Ca^{2+}]_i$ was observed in this study, although our findings agree with those of Flavahan et al. (1989) who

found that ADP-induced endothelium-dependent relaxation was unaffected by pertussis toxin treatment.

Not all endothelial agonists are insensitive to the actions of pertussis toxin: for example, EDRF production induced by α_2 -adrenoceptor agonists, serotonin, histamine and endothelin-3 is regulated by a pertussis toxin-sensitive pathway, as is endothelin-3-induced calcium mobilisation and histamine-induced phosphoinositide hydrolysis (Weinheimer & Osswald, 1989; Flavahan et al., 1989; Voyno-Yasenetskaya et al., 1989b; Emori et al., 1991). Clearly, control of endothelial function may be regulated by at least two different groups of G proteins, only one of which is sensitive to the actions of pertussis toxin.

6.6 Conclusion

In conclusion, this study has demonstrated the ability of a number of agonists to induce biphasic elevations of $[Ca^{2+}]_i$ in bovine aortic endothelial cells. These elevations of $[Ca^{2+}]_i$ are dependent upon mobilisation of $[Ca^{2+}]_i$ from both intracellular and extracellular calcium pools. The intracellular pool consists of two pools - one which is rapidly depleted upon removal of extracellular calcium and a second which is highly resistant to such depletion. Whether these pools are related to the distinct intracellular calcium pools, described by other workers, requires further investigation.

Activation of protein kinase C, by the tumour-promoting phorbol esters, inhibits agonist-induced calcium mobilisation in bovine aortic endothelial cells and this is likely to be the mechanism by which these compounds inhibit EDRF release. However, differential agonist sensitivity to the actions of the phorbol esters was observed and this may account for the ability of these compounds to inhibit agonist-induced EDRF release to varying degrees. As agonists which induce calcium mobilisation were also found to activate protein kinase C, as shown by the ability of staurosporine to increase $[Ca^{2+}]_i$ when added during the plateau phase of the increase in $[Ca^{2+}]_i$, this enzyme may activate a negative feedback pathway which is involved in the regulation of endothelial calcium mobilisation.

Elevation of endothelial cyclic AMP content was found to augment agonist-induced calcium mobilisation, from both intracellular and extracellular calcium pools. Hence, this cyclic nucleotide may enhance the activation of calcium-dependent processes, such as EDRF production, in the endothelium. In contrast to cyclic AMP, elevation of cyclic GMP levels had no effect on endothelial calcium mobilisation. This suggests that cyclic GMP does not modulate endothelial cell function via the inhibition of calcium mobilisation, at least in bovine aortic endothelial cells.

Role of calcium mobilisation in the regulation of endothelial barrier function

7.1 Agents which inhibit endothelial barrier function

The production of vascular leakage and oedema by inflammatory mediators, such as histamine and bradykinin results mainly from the actions of these agents on the endothelium of the post-capillary venule (Majno et al., 1961; Svensjö et al, 1979) and may result from the ability of these agents to induce endothelial cell contraction and inter-endothelial gap formation (Majno et al., 1969). Alternatively, vascular leakage may be promoted by the actions of activated neutrophils, which release oxygen-derived free radicals and proteolytic enzymes, resulting in endothelial cell injury (Wedmore & Williams, 1981). However, evaluation of endothelial barrier function in vivo is complicated by the actions of other cell types, difficulties in precisely evaluating drug concentrations and haemodynamic effects. Ideally, endothelial cells of the post-capillary venule would be the cells of choice if establishing an in vitro endothelial cell culture model to investigate the changes in barrier function occurring during inflammation. However, isolation of endothelial cells from such small vessels is technically difficult and is also likely to result in a small cell yield. Therefore, in a number of studies, including this one, endothelial cells from umbilical vein and pulmonary artery have been

used to assess barrier function as these cells respond, in a similar manner to post capillary venule endothelium, to a number of inflammatory mediators (Killackey et al., 1986; Rotrosen & Gallin, 1986; Carson et al., 1989; Casnocha et al., 1989; Lum et al., 1989; Minnear et al., 1989).

In this study, endothelial barrier function was evaluated by assessing the passage of an inert, high molecular weight marker (trypan blue-labelled albumin) across monolayers of bovine pulmonary artery endothelial cells, cultured on polycarbonate membrane filters. The basal trans-endothelial transfer of this marker was enhanced by thrombin, a receptor-mediated agonist, and by 4 β -phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C. The observation that thrombin increases vascular leakage and, therefore, macromolecular transfer across the endothelial barrier is supported by a number of in vitro and in vivo studies (Killackey et al., 1986; Minnear et al., 1986, 1989; Lum et al., 1989; Casnocha et al., 1989; Lynch et al., 1990).

Phorbol ester-induced increases in macromolecular transport across pig aortic and bovine pulmonary artery endothelial monolayers have also been previously described in in vitro studies (Gudgeon & Martin, 1989; Lynch et al., 1990). The effects of phorbol esters in the endothelium are thought to be mediated via the activation of protein kinase C, as they are mimicked by synthetic diacylglycerols (Lynch et al., 1990), not mimicked by inactive phorbol esters (Gudgeon &

Martin, 1989; Lynch et al., 1990) and inhibited by H7, an inhibitor of protein kinase C (Lynch et al., 1990). In contrast to these studies, PMA inhibited albumin transfer across monolayers of human umbilical vein endothelial cells (Yamada et al., 1990). The reason for this anomaly is not clear, although protein kinase C may exist as different isoenzymes (Kikkawa et al., 1989) which may mediate different cellular functions in different cells. Therefore, if the distribution of these isoforms differs between endothelial cell types, then the responsiveness of these cells to phorbol esters may vary.

Inflammatory mediators may increase macromolecular transport as a result of endothelial contraction and inter-endothelial gap formation in vivo (Majno & Palade, 1961; Majno et al., 1969). In this study, thrombin and PMA enhanced albumin transport across endothelial monolayers and this is likely to correspond with the ability of those agents to induce a number of morphological changes, including shape change, cell contraction and inter-endothelial gap formation (Laposata et al., 1983; Antonov et al., 1986; Garcia et al., 1986; Killackey et al., 1986; Minnear et al., 1989). These morphological changes may result from the redistribution of endothelial contractile proteins - actin, myosin, vinculin and

-actinin have all been found in the endothelium (Becker & Nachmann, 1973; Herman et al., 1982; Drenckhahn, 1983; Franke et al., 1988). It is likely that actin distribution is a key factor involved in the regulation of the

endothelial barrier as the maintenance of inter-endothelial cell junctions is associated with filamentous actin fibres (F-actin) in sub-plasmalemmal regions and at inter-endothelial junctions (Simionescu et al., 1978; Drenckhahn, 1983; Morel et al., 1990; Schnittler et al., 1990). Inhibition of endothelial barrier function may be associated with alterations in the cellular actin pool; for example, via depolymerisation of F-actin to G-actin (the monomeric form of actin), and the centralisation of actin stress fibres (Rotrosen & Gallin, 1986; Garcia et al, 1986; Minnear et al., 1989). As in vascular smooth muscle, development of endothelial cell contraction may occur via an interaction between actin and myosin (Schnittler et al, 1990). Although these morphological changes may account for enhanced endothelial albumin transfer, other mechanisms cannot be eliminated. Albumin transport may occur via vesicular transcytosis (Ghitescu et al., 1986; Milici et al., 1987), although whether or not this pathway is activated by thrombin or PMA remains to be determined.

Inhibition of endothelial barrier function by intracellular messengers may involve elevation of cytosolic calcium ($[Ca^{2+}]_i$) and activation of protein kinase C. An important role for calcium has been suggested by a number of studies. For example, increased vascular permeability, endothelial shape change and endothelial cell contraction are inhibited by removal of extracellular calcium, blockade of calcium influx or buffering of cytosolic calcium (Liddell et al., 1981; Shasby et al., 1985; Lum et al., 1989; Morel et al.,

1989), but promoted by calcium ionophores (Shasby et al., 1985; Olesen, 1987; Gudgeon & Martin, 1989; Morel et al., 1990; Yamada et al., 1990). However, ionophore-induced vascular leakage may simply result from endothelial cell detachment from the substratum (Gudgeon & Martin, 1989). Release of calcium from intracellular stores may contribute to vascular leakage, as discharge of these stores via "caged" $\text{Ins}(1,4,5)\text{P}_3$, induces endothelial cell contraction by a mechanism that is sensitive to calmodulin antagonists (Morel et al., 1990).

Calcium may exert its actions by modulating cytoskeletal proteins. A possible site of action is promotion of actin-myosin induced contractions (Schnittler et al., 1990) via activation of myosin light-chain kinase (Wysolmerski et al., 1990). Calcium-dependent activation of gelsolin (Yin & Stossel, 1979) may promote dissolution of F-actin fibres which, as described above, play a key role in the maintenance of cell shape.

Inhibition of endothelial barrier function can, however, occur in the absence of changes in $[\text{Ca}^{2+}]_i$. For example, in this study, the phorbol ester, PMA, enhanced albumin transfer, but did not elevate $[\text{Ca}^{2+}]_i$. Furthermore, PMA was found to inhibit thrombin-induced elevations of $[\text{Ca}^{2+}]_i$, demonstrating that protein kinase C acts as a bidirectional regulator of cell function. This enhanced albumin transfer may result from endothelial cell contraction and changes in cell shape which have been

previously reported, following exposure to phorbol esters (Antonov et al., 1986; Gudgeon & Martin, 1989). Inflammatory mediators, such as thrombin, histamine and bradykinin may activate protein kinase C via hydrolysis of phosphatidylinositol and its related metabolites (Derian & Moskowitz, 1986; Brock & Capasso, 1988; Pollock et al., 1988) or, alternatively, via hydrolysis of phosphatidylcholine (Martin & Michaelis, 1988). In support of this proposal, recent studies have demonstrated that thrombin induces activation of protein kinase C in bovine pulmonary artery endothelial cells (Lynch et al., 1990).

Protein kinase C enhances the calcium-sensitivity of smooth muscle contractile filaments to calcium (Itoh et al., 1988), although it is not known whether a similar mechanism regulates endothelial cell contraction. Thrombin-induced alterations in endothelial F-actin distribution and content (Garcia et al., 1986; Minnear et al., 1989) may result from increased protein kinase C activation, as phorbol esters have been found to induce actin reorganisation in a kidney epithelial cell line (Schliva et al., 1984). A number of other cytoskeletal proteins (talin, vinculin, vimentin) may also serve as substrates for protein kinase C (Werth et al., 1983; Litchfield & Ball, 1986; Huang et al., 1988b), although it remains to be determined whether protein kinase C acts via these proteins in the endothelium.

7.2 Agents which enhance endothelial barrier function

7.2.1 Cyclic AMP

β -adrenoceptor agonists are known to inhibit vascular leakage induced by inflammatory mediators, at the post-capillary venule (Svensjö et al., 1977, 1979; Marciniak et al., 1978), by a mechanism which may involve direct enhancement of the endothelial barrier. As these agonists elevate endothelial cyclic AMP levels (Hopkins & Gorman, 1981), this cyclic nucleotide was thought to be involved in the regulation of barrier function.

Forskolin, which directly activates the catalytic subunit of adenylate cyclase (Seamon et al., 1981) is known to elevate cyclic AMP content in the endothelium (Adams Brotherton et al., 1982). In this study, it was demonstrated that forskolin enhances the barrier function of bovine pulmonary artery endothelial cells. Elevation of cyclic AMP tended to reduce basal albumin transfer across BPAEC monolayers, although not significantly. Elevated levels of cyclic AMP have been reported to reduce control vascular permeability in a number of studies (Casnocha et al., 1989; Carson et al., 1989), although not in others (Kempski et al., 1987). These differences may simply reflect variable degrees of "leakiness" in different endothelial experimental systems. In this study, elevation of cyclic AMP by forskolin abolished thrombin - and PMA-induced albumin transfer across monolayers of bovine pulmonary artery endothelial cells. These observations are supported by other studies demonstrating the ability of cyclic AMP to inhibit macromolecular transport induced by

histamine, thrombin and PMA across endothelial monolayers (Killackey et al., 1986; Gudgeon & Martin, 1989; Carson et al., 1989; Casnocha et al., 1989). Elevation of cyclic AMP also attenuates thrombin-induced vascular leakage in the lung circulation during in vivo and ex vivo studies (Minnear et al., 1986; Horgan et al., 1987). In contrast to the above studies, Antonov and co-workers (1986) observed increased shape change in human umbilical vein endothelial cells exposed to forskolin, although why the findings of this study differ from all other studies is not clear.

Cyclic AMP-mediated enhancement of endothelial barrier function is associated with a number of morphological changes including a reduced number of endothelial gaps, and the relaxation and flattening of cells (Laposata et al., 1983; Bensch et al., 1983; Minnear et al., 1989; Oliver, 1990). The precise mechanism by which cyclic AMP acts is not entirely clear, although a direct cytoskeletal effect may be involved as its actions are associated with increased F-actin, especially in plasmalammal regions (Minnear et al., 1989; Stelzner et al., 1989). Inhibition of shape change by cyclic AMP may result from phosphorylation, and reduced calcium sensitivity, of myosin light chain kinase (Conti & Adelstein, 1981; Hathaway et al., 1985), an enzyme implicated in the induction of endothelial cell contraction (Wysolmerski et al., 1990). As described earlier, a change in $[Ca^{2+}]_i$ may be an important trigger for alterations in endothelial barrier

function. Therefore, an examination of whether or not elevation of cyclic AMP content inhibited thrombin-induced calcium mobilisation in BPAEC was undertaken. However, in this study, forskolin-induced elevation of cyclic AMP content had no effect on basal $[Ca^{2+}]_i$ or on the magnitude of the thrombin-induced initial transient elevation of $[Ca^{2+}]_i$ but enhanced the plateau phase of the increase of $[Ca^{2+}]_i$, as described in section 6.3. Hence, in contrast to smooth muscle and platelets, where elevated cyclic AMP content inhibits calcium mobilisation (Meisheri & van Breemen, 1982; MacIntyre et al., 1985b), in the endothelial cell cyclic AMP enhances calcium mobilisation. The ability of cyclic AMP to augment calcium mobilisation has been previously observed in hepatocytes and cardiac cells (Cachelin et al., 1983; Poggioli et al., 1986). The findings of this study, coupled with the inability of cyclic AMP to inhibit histamine-induced intracellular release of calcium in human umbilical vein endothelial cells (Carson et al., 1989), suggests that calcium may not be the primary intracellular stimulus for increased vascular permeability. As both thrombin and PMA increase protein kinase C activity in the endothelium (Lynch et al., 1990), activation of this pathway may be the major pathway for inhibition of endothelial barrier function. Cyclic AMP may exert its actions by antagonising the actions of protein kinase C, although the precise cellular location of its actions is not clear.

7.2.2 Cyclic GMP

EDRF, which mediates vascular relaxation via increased cyclic GMP levels (Rapoport & Murad, 1983), has been proposed to enhance vascular leakage (Chander et al., 1988; Hughes et al., 1990). This local hormone effect of EDRF probably results from its vasodilatory actions increasing hydrostatic pressure in vascular beds. However, it is possible that cyclic GMP could modulate endothelial barrier function directly as has been observed with cyclic AMP, as both cyclic nucleotides relax vascular smooth muscle (Itoh et al., 1985).

In this study, endothelial cyclic GMP content was elevated by atriopeptin II, a known stimulant of particulate guanylate cyclase (Leitman & Murad, 1986; Martin et al., 1988) and, additionally, by the membrane-permeant analogue of cyclic GMP, 8 bromo cyclic GMP. However, elevation of cyclic GMP content had no effect on basal or PMA-induced transfer of albumin across monolayers of bovine pulmonary artery endothelial cells, but abolished that induced by thrombin. In support of these observations, elevation of cyclic GMP content inhibited thrombin-induced transfer of albumin across bovine aortic endothelial cell monolayers (Baron et al., 1989), but had no effect on PMA-induced albumin transfer in pig aortic endothelial cells (Gudgeon & Martin, 1989). The inhibitory action of cyclic GMP is associated with the maintenance of inter-endothelial cell junctions and peripheral cytoskeletal filaments (Baron et al., 1989), and with endothelial cell relaxation (Morel et

al., 1989). Atriopeptin II is unlikely to mediate its actions on barrier function via a cyclic GMP-independent mechanism, as its effects were mimicked by 8 bromo cyclic GMP.

The cyclic GMP-mediated relaxation of vascular smooth muscle and inhibition of platelet aggregation, results from inhibition of phosphatidylinositol - (4,5)-bisphosphate (PtdIns(4,5)P₂) breakdown and the subsequent calcium mobilisation in these systems (Takai et al., 1981; MacIntyre et al., 1985b; Collins et al., 1986). Furthermore, elevation of smooth muscle cyclic GMP content may result in inhibition of calcium influx through both VOCs and ROCs (Collins et al., 1988). As described in section 7.1, calcium may be an important stimulus for increased vascular permeability. However, cyclic GMP does not inhibit thrombin-induced albumin transfer in bovine pulmonary artery endothelial cells by inhibiting calcium mobilisation, as neither atriopeptin II nor 8 bromo cyclic GMP inhibited thrombin-induced increases in $[Ca^{2+}]_i$. Both agents induced a small, delayed elevation of $[Ca^{2+}]_i$ when added during the plateau phase of the increase of $[Ca^{2+}]_i$ induced by thrombin. The mechanism involved is not known, although it may involve the inhibition of thrombin-induced protein kinase C activity. Hence, although we cannot eliminate the possibility that cyclic GMP enhances barrier function by inhibition of a calcium-dependent process, it is more likely to exert its actions by inhibiting the protein kinase C signalling pathway. As cyclic GMP had no

effect on PMA-induced albumin transfer, it is clear that it does not inhibit the actions of protein kinase C and, therefore, may block the pathway linking the thrombin receptor to protein kinase C. Support^{for} this proposal is the finding that endothelin-1-mediated activation of protein kinase C is inhibited in rat aorta by elevation of cyclic GMP (Lang & Lewis, 1991b). The inability of cyclic GMP to inhibit calcium mobilisation in bovine pulmonary artery endothelial cells suggests that diacylglycerol (DAG) production from $\text{PtdIns}(4,5)\text{P}_2$ is unaffected, although Lang and Lewis (1991a) have observed a cyclic GMP-mediated inhibition of $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis in pig aortic endothelial cells. Phosphatidylcholine may be an alternative source of DAG in the endothelial cell (Martin & Michaelis, 1988) and its hydrolysis may be important for sustained production of this metabolite. Therefore, if hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ and phosphatidylcholine are regulated by different coupling mechanisms (for example, different G proteins), then selective inhibition could occur.

7.3 Conclusions

In conclusion, this study has demonstrated that the barrier function of bovine pulmonary artery endothelial cells is inhibited by thrombin, a receptor-mediated agonist, and PMA, a direct activator of protein kinase C. PMA enhances albumin transfer in the absence of changes in $[\text{Ca}^{2+}]_i$, suggesting that protein kinase C may have a key role in the regulation of endothelial barrier function and that

elevation of $[Ca^{2+}]_i$ is not a pre-requisite for enhanced macromolecular transfer. Thrombin-induced macromolecular transfer may result from its ability to elevate $[Ca^{2+}]_i$, activate protein kinase C, or from a synergistic interaction between the two messenger systems, as observed in platelets (Rink et al., 1983). As protein kinase C activation also inhibits agonist-induced elevations of $[Ca^{2+}]_i$, this enzyme acts as a bidirectional regulator of endothelial cell function - promoting cell activation while simultaneously inhibiting initiation of subsequent cellular responses.

Elevation of cyclic AMP content inhibits thrombin - and PMA-induced albumin transfer. However, elevation of cyclic AMP content does not inhibit the thrombin-induced increase in vascular permeability via the inhibition of calcium mobilisation and, in fact, enhances calcium mobilisation. Therefore, cyclic AMP may enhance barrier function by inhibiting the intracellular actions of protein kinase C, although we cannot eliminate the possibility that cyclic AMP inhibits the actions of thrombin by inhibiting a calcium-dependent pathway.

Elevation of cyclic GMP content does not inhibit PMA-induced albumin transfer and, therefore, does not inhibit the endothelial actions of protein kinase C. Elevation of cyclic GMP content does inhibit thrombin-induced albumin transfer, but not via inhibition of calcium mobilisation and, therefore, cyclic GMP may

inhibit either a step subsequent to calcium mobilisation or, alternatively, the coupling of the thrombin receptor to activation of protein kinase C.

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